Adulterants and contaminants in herbal supplements sold in Accra (Ghana) for improved sexual performance and their associated health risks

Felicia Akuamoa

Propositions

1. Relying only on chemical analysis for the detection of adulterants in plant supplements sold for sexual improvement is inadequate.
   (this thesis)

2. The advocacy of food fraud especially in the field of herbal supplements endorsed for sexual improvement is unsatisfactory.
   (this thesis)

3. The use of drones in developing countries for the delivery of medical supplies results in a negative cost-benefit outcome.

4. Artificial intelligence (AI) cannot replace common sense.

5. Politicians and managers are insufficiently aware of Walter Cronkite's statement: "Whatever the cost of libraries, the price is low compared to that of an ignorant nation".

6. Emerging threats of digitalization on social and economic growth in Africa far outweigh its benefits.

Propositions belonging to the thesis, entitled

Adulterants and contaminants in herbal supplements sold in Accra (Ghana), for improved sexual performance and their associated health risks.

Felicia Akuamoa

Wageningen, 9 February 2022

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Thesis

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Chapter 1

General Introduction

1.1 Description and aims of the thesis

Plants provide a basic need of man when used as food, nonetheless, their therapeutic roles have also been well recognized. The exploitation of plants for therapeutic purposes due to their active constituents is a global phenomenon which has existed for decades even after the discovery of synthetic alternatives (WHO, 2019). In about 80% of developing countries, the use of medicinal plants forms an integral part of the health-care system due to affordability, the ease in accessibility, engrained socio-cultural practices, strong media persuasion, and 'acclaimed' fewer to no adverse health effects (WHO, 2003). In Ghana, more than 70 % of its citizens use some form of herbal medicine for the treatment and/or management of over 42 diseases and ailments (Asase and Boadu, 2017). In other instances, plants products are used as supplements to complement the nutritional needs of consumers in order to maintain or improve health (Abbiw, 1990; Sanzini et al., 2010; Gyasi et al., 2011).

Although the advantages linked to the use of plants are well recognized, challenges concerning their safety as a result of lack of proper quality control, absence of scientific evidence supporting the acclaimed benefits, the presence of adulterants and other undesirable contaminants of concern cannot be over-emphasized. That notwithstanding, consumers are more focussed on the benefits of these products and the possible solutions they provide to their problems than on the potential adverse effects on their health. One of such herbal products in Ghana are those sold for improving sexual performance which currently some youth and senior citizens have accepted too well. The aim of this thesis was to investigate herbal supplements sold in Accra (Ghana) for the purposes of improving sexual performance, to determine their safety, i.e. to determine their PDE-5 inhibition potentials, identify the potential presence of adulterants and contaminants as well as to assess the associated health risks of consumers.

It is reported that aging, and other activities that result in mental and emotional instability (i.e. stress) including certain chronic medical conditions (diabetes, hypertension), can negatively impact on an individual's sexuality and may potentially result in a dysfunction in their sexual

abilities (Pritzker 1999; Braun et al., 2000; Roumegère et al., 2001; Burnett et al., 2006; Musicki et al., 2009; Lewis et al., 2010). Although sexual dysfunction varies significantly between males and females, erectile dysfunction (ED) which is the inability of a man to achieve and maintain an erection for a satisfactory sexual performance, is global issue projected to affect over 320 million men especially in Africa, Asia and South America by the year 2025, thus has gained the necessary attention over the past two decades (Ayta et al., 1999; Moreland, 2000; Schramek et al., 2014).

Different solutions ranging from psychological therapies (counseling) to prescriptive drugs have been approved for the treatment and or management of ED since the early 1990s (Patel et al., 2014). Approved drugs often recommended for the treatment of ED are known as phosphodiesterase type 5 inhibitors (PDE-5i). PDE-5i work by inhibiting the degradation of cyclic guanosine monophosphate (cGMP) by PDE-5 enzymes in the smooth muscle cells (Ballard et al., 1998; Moreland 2001). In so doing, the corpora cavernosa of the penis is dilated and enlarged, permitting enough blood flow to the penis and subsequently enhancing erection. Examples of approved PDE-5i includes, sildenafil citrate, sold under the brand name (Viagra), tadalafil (Cialis), vardenafil hydrochloride (Levitra), and avanafil (Stendra). There are also plant-based sexual enhancers especially in most African countries which are highly preferred by consumers although they are not medically approved or scientifically proven to be safe. However, due to the high cost and adverse effects of synthetic PDE-5i, these herbal alternatives are much preferred (Gresser, U. & Gleiter, C. H. 2002; Lowe et al., 2012; Ventimiglia et al., 2016).

In Ghana, due to the high cost of medical consultation and the assumption that natural products are generally 'safe', most individuals experiencing sexual challenges, self-medicate by employing available herbal remedies accessible from various outlets (Afrane et al., 2016). Although the majority of these products are locally produced, some are imported, while others are smuggled in from neighbouring countries due to the porous nature of the border systems.

These herbal sexual enhancers are not only popular amongst individuals having sexual challenges (e.g. ED) but also amongst the youth who employ them for recreational purposes. A study by

Danquah et al. (2011) revealed that 61% of males and 48% of females in some parts of Ghana, use some form of sexual enhancing supplement to improve their sexual abilities. Although they are marketed and promoted as 'all natural', the majority are reportedly adulterated with active pharmaceutical ingredients (APIs), which includes PDE-5i and/or their analogues (Reeuwijk et al., 2013; Patel et al., 2014; Bujang et al., 2017; Gilard et al., 2018).

Several conventional and emerging chromatographic and mass spectrometric techniques are efficiently employed in the identification, quantification and/or structural elucidation of known and unknown APIs commonly used in adulterating herbal products (Gratz et al., 2004; Petal et al., 2014). However, due to the on-going production of new analogues, these existing techniques may not detect and identify new compounds, especially when they are not available in the reference library. Currently, the use of a tiered approach (where a biological assay is first used to screen and semi-quantify unknown bioactives in botanicals, followed by chemical analysis using various chromatographic and mass spectrometric techniques) for the identification and quantification of compounds in complex mixtures (i.e. botanicals) are becoming popular with minimum challenges (Santillo and Mapa, 2018; Fu et al., 2020). This combined approach was employed in this thesis, where the PDE-Glo and the Dioxin Responsive Chemical-Activated LUciferase gene eXpression (DR CALUX®) assays were initially used in screening samples followed by chemical analysis using various chromatographic techniques (i.e. LC-MS/MS and NMR analysis) for the identification and quantification of active compounds.

In this thesis, apart from determining the inhibition potentials of selected supplements to assess their safety, the presence of adulterants and other contaminants, such as polycyclic aromatic hydrocarbons (PAHs), dioxins and dioxin-like polychlorinated biphenyls (dl-PCBs), including pyrrolizidine alkaloids (PAs) in the selected supplements, which represent plant constituents of concern were also investigated. The presence of PAHs, dioxins and dl-PCBs may be the results of processing methods used (i.e. drying over open fires), whiles PAs may be present as natural constituent of plant(s), or co-harvesting of PA-containing weeds when collecting the raw materials,

or adulteration of non-PA-containing raw materials with PA-containing plants. The presence of PAHs, dioxins and dl-PCBs raises concerns due to their binding affinity to the aryl-hydrocarbon receptor (AhR) which may potentially alter the cells' normal function (Denison M.S. and Nagy S.R, 2003; Bradfield et al., 2009). The 1-2 unsaturated PAs and certain PAHs are considered genotoxic and carcinogenic compounds by the International Agency for Research on Cancer (IARC, 2003) due to their metabolic activation in the liver and subsequent formation of highly reactive pyrroles and diol epoxides respectively, which may interact with the genetic material (i.e. DNA) thereby interfering with its normal functions and introduction of errors in their replication.

1.2 Role of phosphodiesterase type-5 (PDE-5) enzymes in the smooth muscle cells and the mode of action of PDE-5i

Phosphodiesterases are a group of enzymes numbering 1 to 11 (i.e. PDE-1 to 11), and are responsible for the regulation of cellular levels of second messenger molecules (i.e. cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP)) (Conti M and Beavo J, 2007). PDE type-5 enzymes are predominant in the smooth muscle cells and are responsible for the regulation of cGMP levels, thereby promoting the contraction (linked to reduced levels of cGMP due to degradation) or relaxation (due to accumulation of cGMP initiated by the release of nitric oxide (NO) from the nerves, vascular endothelium and smooth muscle cells) of this muscle (Burnett A. L., 1997). PDE-5i inhibit the degradation of cGMP by PDE-5 enzymes at the active catalytic sites of the enzymes-substrate complexes, thereby permitting the accumulation of cGMP to levels needed for smooth muscle relaxation, thus resulting in improved blood flow to the penis and subsequent enhancement of erection (Corbin et al., 2000).

1.3 Contaminants in plant supplements and their associated health risks

In this thesis the presence of several constituents of concern in herbal supplements sold for improving sexual performance was investigated, providing the foundation for accompanying risk assessment. In the next sections, the investigated constituents of concern are introduced to some further extent .

1.3.1 Adulteration of herbal supplements with PDE-5i

Various plants such as horny goat weed (known to contain Icariin), Thai ginseng, panax ginseng, rhodiola rosea and ginkgo biloba have been purported to contain natural compounds with PDE5 inhibition potentials (Singh et al., 2012; Chaiyakunapruk et al., 2016; Ganapathy et al., 2020). However, the issue is whether this is really the case and if the levels are high enough to cause the desired effect, also in relation to the relative potencies of the responsible compounds. Furthermore, the efficiency of preparations derived from these plants often do not meet the expectations of consumers. This is partly due to the dynamic nature of plant properties with the levels of constituents of interest known to be dependent on age of the plant, plant part used, time and season for collection, correct species, environmental/climatic conditions, incidence of drought/ temperature stress, and effects of pests and diseases (Abdillahi et al., 2012). In addition, processing methods including storage, and mode of administration play important roles in the levels of active constituents and consequently the plants' therapeutic efficiency. Ultimately, the efficacy of these plant products are also influenced by the pharmacokinetic and pharmacodynamic differences amongst individuals.

All these factors may prevent products from working efficiently to the disappointment of users. Producers, in an attempt to address these challenges, may add approved synthetic PDE-5i (e.g. sildenafil, vardenafil hydrochloride, tadalafil, or avanafil) and/or their analogues (i.e. homosildenafil, nortadalafil, hydroxyvardenafil) (Fig. 1) to these products at pharmacological relevant levels in order to achieve the desired outcome (Singh et al., 2009; Reeuwijk et al., 2014; Malet-Martino et al., 2018).

Approved PDE-5i are prescription-only drugs used exclusively upon medical recommendation as first line treatment for ED with appropriate monitoring. Most of their analogues are illegally produced by some manufacturers mostly for adulteration purposes. The use of PDE-5i are strongly contraindicated in patients using organic nitrate drugs, α-adrenergic blockers and CYP3A4 inhibitors (Kostis et al., 2005; Langtry and Markham, 1999). The combined intake of any of these

drugs and PDE-5i may synergistically promote increased relaxation of smooth muscle cells, resulting in a drastic reduction in systemic blood pressure, which may possibly lead to other cardiovascular effects and in extreme cases, sudden shock or even death (Langtry and Markham, 1999; Kostis et al., 2005; Kloner, 2007; Gur, et al. 2013). Patients who are advised against PDE-5 inhibition drugs may resort to herbal alternatives due to the belief that they can innocuously induce the same pharmacological effect while circumventing the expected adverse effects (Blok-Tip et al., 2004). However, due to the high prevalence of adulteration of these products, consumers may unknowingly expose themselves to unanticipated risks.

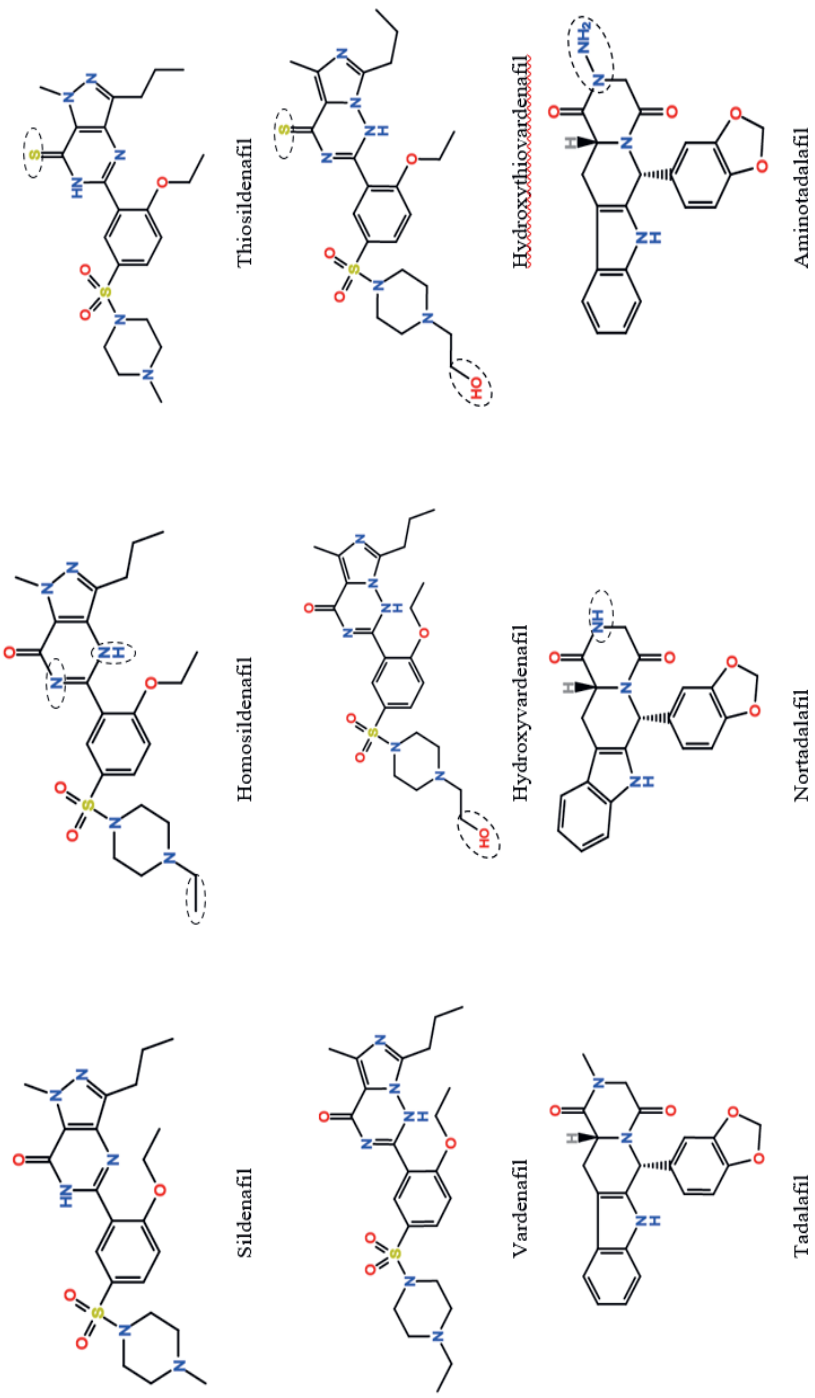

Fig. 1: Some approved PDE-5 inhibitors and their analogues. Dotted circles indicate the modified part of the parent structure in the analogues.

1.3.2 Pyrrolizidine alkaloids

The journal of plant physiology and pathology defines plant metabolism as 'a complex of physical and chemical events of photosynthesis, respiration, and the synthesis and degradation of organic compounds'[1]. These organic compounds includes secondary plant metabolites, with specific roles involving the protection of plants from injuries and predators (pests and pathogens), and support plants in adapting to their environment. According to Bourgaud et al. (2001), secondary plant metabolites are classified according to their biosynthetic pathways and therefore are grouped under three molecular families (i.e. alkaloids, phenolics, and terpenes and steroids). Pyrrolizidine alkaloids (PAs) are a group of naturally occurring alkaloids mainly produced by certain plants for defense, and are found in more than 12 higher plant families of which three (i.e. Compositae (Asteracae), Boraginaceae and Leguminosae (Fabaceae)) are considered the most toxic (Gerardo I., 2005).

PAs have the structure of pyrrolizidine (two structured five-membered rings with a shared nitrogen atom) and occur as esters of a necine base (amino alcohols) with one or more necic acids (mono- or dicarboxylic aliphatic acids), which contributes to the structural differences between the different PAs (Cunha APD., 2010; Pereira et al., 2018) (Fig. 2). Based on these structural differences, PAs are grouped into four classes (i.e. platynecine, retronecine, heliotridine and otonecine type PAs) (Fig 3), which either occurs in their tertiary base or *N*-oxide (PANO) forms. Platynecine is a saturated PA and considered not toxic. However, retronecine-, heliotridine- and otonecine types of PAs are considered most toxic due to the double bond in the 1,2-position, hence the name 1,2-unsaturated PAs.

PAs on their own are unreactive, however upon metabolic activation in the liver by cytochrome P450 (CYP3A and CPY2B6 isozymes) these unreactive compounds may be converted into highly reactive ester pyrroles (electrophiles) which may react with nucleophilic tissue components such as nucleic acids and proteins (Prakash et al., 1999). According to Fu et al. (2002), three principal metabolic pathways are involved in the formation of 1,2-unsaturated PAs. These includes hydrolysis

[1] https://www.scitechnol.com/plant/plant-metabolism.php assessed on 5/08/2021

of the ester functional group resulting in the necine bases (i.e. heliotridine-, retronecine-, and otonecine-type), oxidation of the necine bases to their respective *N*-oxides, and hydroxylation at C-3 or C-8 position of the necine base resulting in 3-, or 8-hydroxynecine derivatives and subsequent dehydration to form the corresponding dehydro-pyrrolizidine (pyrrolic) derivatives. The formation, bioactivation and mechanism of action of PAs have been extensively discussed in different reviews and other studies (Prakash et al., 1999; Fu et al., 2002; Pereira et al., 2018; Hessel-Pras et al.,2019). The liver is the primary site for the production of toxic pyrroles, making it one of the two target organs aside the lungs. The injury caused by these toxic metabolites in the hepatic veins is what leads to veno-occlusive disease (VOD), also known as hepatic sinusoidal obstructive syndrome (HSOS) (Prakash et al., 1999) with symptoms such as vomiting, enlargement of the liver and bleeding diarrhea (Chen et al., 2010).

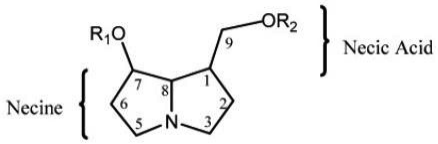

Fig. 2: Basic structure of a PA

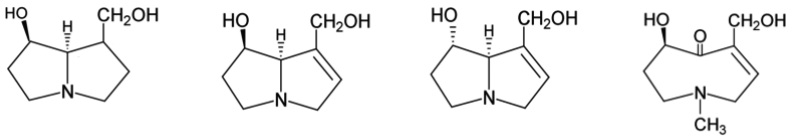

Fig.3: Groups of PAs according to the type of necine base

Intoxication by PAs can be acute, sub-acute or chronic. These are characterized by hemorrhagic necrosis, hepatomegaly and ascites (acute), blockage of hepatic veins leading to hepatic sinusoidal obstructive syndrome (HSOS) (sub-acute) and necrosis, fibrosis, cirrhosis and proliferation of bile duct epithelium and in extreme cases, liver failure, liver cancer and even death (chronic) (Helmy A., 2006; Chen and Huo, 2010; Biavatti et al., 2013). One detoxification pathway of these highly reactive intermediates is through conjugation with glutathione (Ruan et al., 2014). Although, this detoxification pathway has been shown to reduce PA-induced cytotoxicity (Ji et al., 2009; Neuman

et al., 2007), a study by He et al. (2017) revealed that PA-derived glutathione adducts may equally bind to cellular DNA and cause toxicity.

1.3.3 Polycyclic aromatic hydrocarbons (PAHs)

PAHs are a group of chemicals consisting of more than 200 organic compounds containing carbon and hydrogen with two or more single or fused aromatic rings arranged in a linear, clustered or angular form. PAHs with up to and including six fused aromatic rings are known as “small” PAHs, while those with more than six aromatic rings are considered “large” PAHs (Abdel-Shafy and Mansour, 2016). As the number of fused rings increases, so does their molecular size, lipophilicity, environmental persistence and the degree of damage to genetic material induced upon bioactivation (Cerniglia C.E., 1992). Various environmental and human activities such as forest fires, coal burning, vehicle exhaust emissions, improper burning and cigarette smoke contribute to their occurrence in the environment (Wenzel T. and Zelinkova Z., 2015). Three fundamental pyrolytic conditions (i.e. high temperatures, reduced oxygen levels, and presence of organic matter) supports their generation. Exposure through food is mainly the result of the processing methods used (i.e. drying over open fires, roasting, grilling or smoking), where organic compounds are partially degraded into smaller unstable fragments (pyrolysis) which recombine to produce relatively stable PAHs (Lawrence and Weber 1984; Harvey 1997; Howsam M. and Jone K., 1998; Moret and Conte 2000).

PAHs has been classified as priority pollutant by the IARC, and the United States Environmental Protection Agency (USEPA) due to their genotoxic and carcinogenic potentials. The Codex Alimentarius Commission indicated that the high reactivity of PAHs is due to the presence of Bay and Fjord regions (Orecchio, Ciotti, and Culotta, 2009) (Fig. 4). These PAHs are converted to diol epoxides that act by covalently binding with DNA, thereby adding errors in their replication (mutations) and initiating the formation of tumors (Cerniglia C.E., 1992; Billiard et al., 2002). The EU Scientific Committee on Food (SCF) in 2002 identified 15 PAHs as being of concern to human health due to their carcinogenicity. However, EFSA in 2008, reviewed the available occurrence data and concluded that the sum of 4 PAHs (Σ4PAHs), i.e. benzo[*a*]anthracene, benzo[*a*]pyrene,

benzo[*b*]fluoranthene and chrysene, is the most suitable indicator for genotoxic PAHs in food. Studies have shown a correlation between PAH exposure and cancers of the respiratory system, stomach and enhanced risk of renal cell carcinoma upon intake of grilled meat (Daniel et al., 2011; Ledesma et al., 2014).

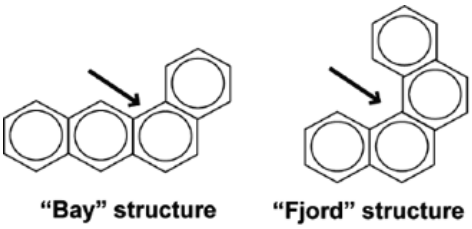

Fig. 4: PAHs with Bay and Fjord region

The adverse effects of PAHs on human health largely depend on factors such as the duration, route of exposure, dose/amount of PAHs the individual is exposed to, and the relative toxicity of the particular PAHs (Abdel-Shafy and Mansour, 2016). Other individual factors such as pre-existing health status and age equally play important roles.

Short-term health effects (acute)

Exposure to high levels of PAHs in a mixture of compounds have resulted in symptoms like eye irritation, nausea, vomiting, diarrhea, confusion, skin irritation and inflammation (Unwin et al., 2006). PAHs such as anthracene and benzo(a)pyrene are both known skin irritants (Abdel-Shafy and Mansour., 2016).

Long-term health effects (chronic)

Chronic exposure to PAHs includes genotoxic and carcinogenic effects (Scientific Committee on Food (SCF) (EC, 2002)), immune suppression, cataracts, breathing problems with asthma-like symptoms, abnormalities in lung function, kidney and liver damage. Naphthalene in particular, when inhaled or ingested in large amounts can cause breakdown of red blood cells (Abdel-Shafy and Mansour., 2016).

1.3.4 Dioxins and dioxin-like polychlorinated biphenyls

Polychlorinated dibenzo-*p*-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs) are accidentally produced through burning of some materials and certain industrial or manufacturing processes (IARC, 1993; Ahlborg et al., 1994; Hanano et al., 2015). Their presence in plant products is mainly the result of contaminated raw materials harvested from highly polluted areas. The PCDD/Fs of concern are composed of two benzene rings with chlorine substituents (four to eight) linked together by one or two oxygen atom(s), respectively. Compounds referred to as dl-PCBs are those with structural similarity and mode of action (i.e. binding to the aryl hydrocarbon receptor (AhR) as PCDD/Fs (Nebert et al., 1993; White and Birnbaum, 2009). They are present in commercial PCB-mixtures that are no longer produced, however, due to their ubiquitous nature, PCBs are widespread environmental contaminants. The affinity at which PCDD/Fs bind to the AhR determines their potency. Also, certain non-ortho-substituted PCBs are noted for their high binding affinity to the AhR (Safe et al., 1985; Van den Berg et al., 2006). Therefore, risk assessment of these compounds often involves the evaluation of complex mixtures of PCDD/Fs and dl-PCBs sharing a similar mode of action (MOA), persistency and assumed dose additivity (a key underlying assumption behind toxic equivalency factors (TEF) (Birnbaum, 1999; Behnisch et al., 2003; van den Berg et al., 2006).

TEFs are established based on a variety of biological endpoints which are dependent on the magnitude of effects induced by individual congeners compared to the index compound. As confirmed by Haws et al. (2006), 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), with substituted chlorine atoms in position 2, 3, 7 and 8, has the strongest binding affinity to the AhR and is thus considered the most toxic congener. Based on this report, the WHO (2005) considered 2,3,7,8-TCDD as the index chemical and assigned to this compound a TEF value of 1. Subsequently, the relative potencies for six other PCDDs, ten PCDFs and twelve dl-PCBs with lower binding affinity relative to 2,3,7,8-TCDD, were expressed as a fraction of the potency of 2,3,7,8-TCDD (to cause the same effect), to derive their TEF values. In other words, the ratio of the half maximal effective dose (ED_{50}) of dioxins and dl-PCBs divided by the ED_{50} of TCDD is the basis for the assigned TEF

value of the respective congener (Kodavanti et al., 2017). In the analysis of food and feed, the concentration in the mixture for each congener is multiplied by its TEF value to derive the contribution of that congener to the total toxic equivalency (TEQ). So, the total TEQ (presented as WHO-TEQ) expresses the toxicity in TCDD equivalents as if the mixture contained only TCDD (Dickson and Buzik, 1993; Fries, 1995; Safe, 1998). This TEF-concept has been developed and accepted by international organizations (i.e. The United States Environmental Protection Agency (USEPA), The European Food Safety Authority (EFSA), and the WHO) to facilitate risk assessment and regulatory control of PCDD/Fs and dl-PCBs.

The relative response of dioxins and dl-PCBs in the DR CALUX® assay reflects the TEF-values. Still, Hoogenboom et al. (2010) argued that, because the DR CALUX® assay may also detect other non-regulated AhR-agonists not included in chemical analysis, the outcome should be regarded as a qualitative feedback or semi-quantified result and therefore the units should be expressed in bioanalytical equivalents (BEQs) rather than TEQs. This is also described in the EU-legislation regarding the required performance criteria.

1.4 The Aryl hydrocarbon Receptor (AhR)

One of the key toxic effects of PAHs, PCDD/Fs and dl-PCBs, is their binding affinity to the aryl hydrocarbon receptor (AhR) and subsequent induction of the AhR-mediated pathways. The AhR is a cytosolic multiprotein complex belonging to a subclass of helix–loop–helix-containing transcription factors known for its role in the toxicity of xenobiotics in cells (Goldstein J. A., 1989; Giesy and Kannan, 1998). The AhR is located in the cytoplasm in a complex with its chaperones including a heat shock protein 90 kDa (Hsp90), protein 23-kDa (p23) and the aryl hydrocarbon receptor associated 9 protein (AhRA9) also known as X-associated protein 2 (XAP2) (Denison M.S. and Nagy S.R, 2003). The hsp90 holds steady the AhR in the cytoplasm in order to prevent its translocation to the nucleus. P23 stabilizes the interaction between the AhR and the Hsp90 and the AhRA9 protein enhances AhR signaling through its proper folding in the cytoplasm (Poellinger et al., 2000; Bradfield et al., 2009). The AhR complex, upon ligand binding, translocate to the cell's nucleus, where it dimerizes with the aryl hydrocarbon receptor nuclear translocator (AhRNT), thus

converting the AhR-ligand complex into its high affinity DNA binding form (the form in which it binds to dioxin-responsive elements (DREs) in the DNA), thereby initiating the transcription of adjacent genes (Denison M.S. and Nagy S.R, 2003; Bradfield et al., 2009). Fig. 5 summarizes the AhR-ligand association and the mechanism of activation (adopted from Denison M.S. and Nagy S.R, 2003).

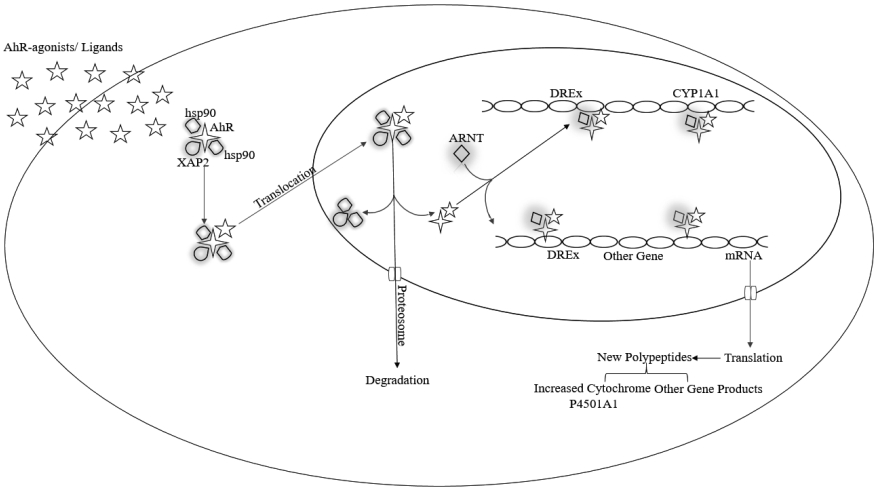

Figure 5: Aryl hydrocarbon receptor (AhR) signaling pathway

1.5 Analytical methods employed in this thesis

1.5.1 Chemical analysis

Analytical methods are employed for monitoring purposes, but also for research, product development and quality control. Areas include for instance, food and feed products, pharmaceuticals, customer products like baby toys, but also environmental and forensic samples. The selection of an appropriate analytical method is crucial as it guarantees the reliability and success of the analysis. The experimental procedure may start with a more or less destructive method for sample preparation prior to analysis by various chromatographic and/or spectroscopic techniques for the identification, quantification, characterization and or structural elucidation of compounds of interest. Several conventional techniques involving extraction, filtration, centrifugation, evaporation, and sublimation are being employed in the separation, qualitative and quantitative

assessment of analytes. However, in recent times, the use of chromatography is much preferred due to its ease, automatization, and commercialization (availability of columns), resulting in excellent and sufficient precision in separating different molecules of various sizes present in complex mixtures and thus in high accuracy and specificity. In this thesis, the liquid chromatography (LC) and gas chromatography (GC), both in tandem with triple quadruple mass spectrometry (MS/MS), were the two main separation techniques employed (Siddiqui et al., 2017). Nuclear magnetic resonance (NMR) was also used for structural elucidation of unknown compounds.

Chromatographic techniques may be classified as preparative or analytical based on the principle of separation. The two main phases during chromatographic separation are the 'mobile phase' (consisting of liquid or gas) and the 'stationary phase' (i.e., most often a column consisting of immobilized liquid, solid or gel) (Forgács, E. and Cserháti, T., 2003). During chromatographic analysis, injected mixtures containing the analyte are first dissolved in the 'mobile phase', and subsequently carried through the stationary phase which is a fixed instrument system consisting of a column, capillary tube, a plate, or a sheet (McMurry J., 2011). The duration of attachment of molecules to the stationary phase depends on their physicochemical properties (e.g. size, mass, volume, polarity) influencing the level of affinity for the stationary phase. As a result, slight differences in the partition coefficient of compounds, results in different retention times on the stationary phase, thus resulting in different separations or chromatograms (McMurry J., 2011).

Liquid chromatography (LC) and gas chromatography (GC) are the most widely used separation techniques in analytical chemistry. LC uses liquid in the mobile phase while separation is usually carried out on a column or a plane. In contrast, GC uses gas in the mobile phase while separation is carried out on a column which is either "packed" or "capillary". Liquid chromatography (LC) separates compounds in mixtures using columns containing very small particles (2-5 mm in average) under relatively high pressure (50-350 bar) (Mu Naushad and Khan M.R., 2014). Thus in LC analysis, samples are forced by a liquid under high pressure (mobile phase) through a stationary phase composed of irregularly shaped particles, and a porous membrane. Following separation,

compounds are either determined off-line or via coupling of the column to a relevant and efficient detection system for qualitative and/or quantitative analysis (e.g. detection by UV or MS).

Coupling LC and GC techniques to an on-line spectroscopic detection system, enables the exploitation of the advantages of both techniques; where the chromatographic system separates chemical compounds in a mixture based on their physicochemical properties, while the on-line spectroscopy system acquires relevant data for the identification of eluted compounds by comparing them to standards or library spectra (Patel et al., 2010). When using mass spectroscopy based detection, the separated compounds eluting the column are identified based on their mass spectral (MS) data, providing information on the molecular weight and fragmentation pattern of the compounds. This ultimately aids in the confirmation of a compound's identity and may also facilitate the identification of unknown compounds, although full structural elucidation can often only be achieved by the additional use of nuclear magnetic resonance analysis (NMR).

Structural elucidation of a compound from a single LC-MS run may also be hampered by the soft ionization techniques of the MS system, especially when this results in limited fragments of the compound's molecular ion species (Patel et al., 2010). This can be resolved through the use of tandem mass spectrometry (MS/MS), which provides fragments through a collision-induced dissociation of the molecular ions of the parent compound (Herderich et al., 1997). LC-MS/MS has proved its value especially when used together with screening assays for investigating natural products (Patel et al., 2010).

Technological advancement has led to the development of mass analyzers that permit more efficient sorting of ions according to their mass-to-charge ratios. The time-of-flight (TOF) mass analyzer is a typical example which offers superior advantages such as increased resolving power of signals on the m/z axis, accurate mass measurements (1–2 mmu) of the fragment ions ejected from the collision chamber, resulting in the correct identification of unknowns, including the establishment of empirical formula of fragment ions (Ferrer and Thurman, 2003). The improved resolving power of

the TOF mass analyzer also aids in the accurate measurement of masses of compounds present at extremely low concentrations.

While the separation techniques (LC/GC) are useful for a compound's preliminary identification by its retention time, and the TOF mass analyzer provides an added advantage of determining the accurate mass of compounds, their combination is regarded as a powerful tool for known compounds when enough reference compounds and/or big libraries are available. However, most often additional methods, such as NMR, are needed for the identification of non-target (unknown) compounds. NMR has proved its worth in the structural elucidation of compounds in complex matrices, especially natural products, when present in high concentrations. When present at low levels, preparative separations are needed before NMR analysis can be of assistance. An NMR instrument consists of a superconducting magnet, a spectrometer, a control system, and a detector (probe) which measures the interaction between electromagnetic radiation and materials (Shin-ichi Yusa, 2020). During NMR analysis, the analyte is placed in the magnetic field and irradiated with radio waves from a spectrometer, enabling measurement of the absorption of radio waves by the protons (^{1}H), isotope ^{13}C of carbon or relevant isotopes of other nuclei (Mallakpour S. and Azimi F., 2020). There are various types of NMR, however, the most commonly used techniques are proton (^{1}H-), and carbon (^{13}C-) NMR. ^{1}H NMR determines the type and number of hydrogen (H) atoms in the molecule, while ^{13}C NMR determines the type and number of carbon (C) atoms in the molecule. Although the identification of known active compounds in complex mixtures using chemical analysis often provides adequate information, the combined use of chemical and biological analysis serves a dual purpose of identifying and quantifying bioactives in complex mixtures, both known and unknown compounds, while assessing their total biological response simultaneously (either *in vivo* or *in vitro*). Different bioassays for different endpoints are described, such as the chromosomal aberration test, micronucleus assay, mouse lymphoma tk assay, Ah-immunoassay, ethoxyresorufin-O-deethylase (EROD) assay, and the Ames test which are amongst the popular biological assays commonly used to assess the responses of cells exposed to different genotoxic chemicals. In this thesis, two biological assays were employed to assess the response of cells *in-vitro*. They include

the PDE-Glo bioassay developed by Promega and the Dioxin Responsive Chemical-Activated LUciferase gene eXpression (DR CALUX®) assay.

1.5.2 PDE-Glo Phosphodiesterase Assay

The PDE-Glo™ Phosphodiesterase assay is a high-throughput luminescent screening (HTS) assay involving a series of steps initiated by the incubation of purified phosphodiesterase (PDE-5), a substrate (i.e. cGMP) and an inhibitor (i.e. PDE-5i) for 90 minutes. The remaining cGMP after incubation serves as a catalyst in the conversion of inactive protein kinase A (PKA) to active PKA. The activated PKA phosphorylates PKA substrate, which uses ATP in the process. The amount of ATP remaining after the phosphorylation process, is used to produce light in the presence of luciferin + O_2 (in the Kinase Glo reagent). The intensity of light corresponds with the PDE-5 enzyme activity during the incubation. In summary, the higher the concentration of the inhibitor, the lower the degradation activity of the PDE-5 enzyme, meaning more substrate (cGMP) will be left after the incubation process. High amounts of substrate (cGMP) means more ATP will be used in the phosphorylation process, thereby resulting in less light production.

1.5.3 The DR CALUX® assay

Different methods are used to determine the levels of PCDD/Fs and dl-PCBs in environmental samples as well as in food and feed matrices. A distinction is made between the so-called 'reference method' and the alternative or screening methods. Gas-chromatography (GC) in combination with high resolution mass spectrometry (HRMS) is used as the reference method (i.e. the so-called 'golden standard') for the identification and quantification of PCDD/Fs and dl-PCBs (Behnisch et al., 2001; Liem, 1999). However, bioassays like the DR CALUX bioassay can be used for screening purposes to determine the biological response of cells when exposed to PCDD/Fs and dl-PCBs, including compounds with similar mode of action.

Different assays have been developed for rapid screening of various matrices (food/ feed, sediments, soil) for the presence of PCDD/Fs and dl-PCBs. These include AhR receptor binding assays and cell based transcriptional activation assays that measure the expression of a marker protein or

enzyme (e.g. fluorescent protein, EROD or luciferase). So far, only the cell based DR CALUX® assay, expressing luciferase upon activation of the AhR, has successfully been used for testing of feed and food samples. Although the DR CALUX® assay has its own advantages which includes, the use of relatively small sample size, extreme sensitivity, and reduced cost (relative to chemical analysis), it equally presents some challenges (i.e. lack of specificity leading to false-positive predictions, requirements for extensive sample cleanup, and potential interferences by non AhR-ligands) (Bovee et al., 1998; Behnisch et al., 2002; Prinsloo et al., 2017). Because certain PAHs can stimulate the AhR (EFSA 2008), the DR CALUX®, which was initially developed for the detection of dioxins and dl-PCBs, is also suited for screening for the presence of PAHs.

The DR CALUX® assay is a ligand-dependent nuclear receptor-based bioassay, by which rat (H4IIE) or mouse (H1L6.1) hepatoma cells are stably transfected with a dioxin-responsive element (DRE) sequence coupled to a luciferase reporter gene, such that their exposure to (AhR)-agonists will result in the production of luciferase (Windal et al,. 2005; Hoogenboom et al., 2006). Induction of luciferase is time-, dose- and chemical dependent, where the amount of luciferase activity is directly related to the concentration and potency of the inducing compound (Windal et al,. 2005). Due to the high sensitivity of the DR CALUX® assay, it lacks specificity thus showing responses from all compounds able to stimulate the AhR. Concerns about the lack of sensitivity and specificity of the DR CALUX® assay has been addressed in the review by Windal et al. (2005). They indicated that an acid silica clean-up during sample preparation, as developed by Bovee et al. (1998), improves selectivity for dioxins and dl-PCBs and limits interferences from other AhR-agonists, including naturally occurring compounds like furocoumarins and acid-condensation products of indol-3-carbinol. The more stable and persistent compounds such as PCDD/Fs and dl-PCBs are noted to survive the acid silica clean-up, however, the less stable compounds such as the PAHs and other AhR-agonists are lost. This helps to clearly distinguish between the responses induced by less stable AhR-agonist (e.g. PAHs) with highest response observed for samples without an acid silica cleanup, and that of stable AhR-agonist (e.g. PCDD/Fs and dl-PCBs) with highest response observed for samples with acid silica clean-up). More so, due to the less stable nature of PAHs, cells show

response after a short exposure time. Thus the increased response of cells elicited after short-term exposure (i.e. 4 hrs) enables the identification of less stable AhR-agonist (i.e. PAHs) (Bovee et al., 1996). However, due to the stable nature of PCDD/Fs and dl-PCBs, a longer exposure time is more selective for these compounds, thus resulting in higher responses after 48 hrs exposure. In exceptional cases, less stable AhR-agonists which are in higher concentrations do elicit high responses even after 48 hrs exposure due to metabolic limitation of cells.

1.6 Risk assessment approach

The risks for consumers as a result of exposure to plant constituents of concern upon use of the selected supplements analyzed in the present thesis, were assessed based on the estimated daily intakes (EDIs) in order to predict when risk management actions by relevant authorities would be considered necessary. For PCDD/Fs and dl-PCBs causing adverse effects by a non-genotoxic mode of action (i.e. immune suppression, chloracne, epithelial hyperplasia, teratogenesis), the most critical endpoint was reduced sperm production in men exposed at young age, which was the basis for the establishment of the Tolerable Weekly Intake (TWI) by EFSA in 2018. Tolerable (denoting permissibility and not acceptability) weekly intake in principle presents an estimate of the amount per unit body weight of a potentially harmful substance/contaminant that can be ingested over a lifetime without adverse health risk. Synonymous in context to the TWI is the Tolerable Daily Intake (TDI), but the TWI is preferably used for contaminants that are persistent in the environment and show the tendency to accumulate within the body over a longer period of time. The EFSA Panel on Contaminants in the Food Chain (CONTAM) in its scientific opinion on dioxins and dioxin-like PCBs (2018) took into account reported effects in humans and the exposure from breastfeeding and the twofold higher intake during childhood to establish a TWI of 2 pg TEQ/kg bw/week, using toxicokinetic modelling (EFSA, 2018).

However, for compounds with a genotoxic and carcinogenic mode of action (i.e. PAHs and PAs), EFSA (2005) concluded that no safe threshold value and a related health-based guidance value can be derived. As a result, EFSA indicated that for these compounds, the margin of exposure (MOE) approach is best suited for evaluating the risk and characterizing priorities for risk management

actions. Accordingly, in the present thesis the risks associated with use of the herbal supplements containing PAHs and PAs were characterized based on the MOE approach. The MOE is the ratio between the lower confidence limit of the benchmark dose (i.e. $BMDL_{10}$), derived by mathematical dose-response modelling of experimental animal data, to the estimated human exposure (EDI). The $BMDL_{10}$ is the lower 95% confidence limit for the dose resulting in a 10% increase in tumor incidence above background values.

For PAs, EFSA (2005) established a $BMDL_{10}$ of 237 µg/kg bw/day derived from a study in rats exposed to riddelliine (NTP 2003), whereas in the case of the 4 EU marker PAHs (i.e. benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene and benzo[*a*]pyrene) a $BMDL_{10}$ of 340 µg/kg bw/day was derived by EFSA (2008), based on a carcinogenicity study on coal tar mixtures by Culp et al. (1998). Overall, the EFSA considers an MOE value of 10 000 or higher as indicative of a low concern and therefore a low priority for risk management actions, whereas MOE values below 10 000 are considered priority for risk management actions (EFSA 2005).

1.7 Objectives and outline of the thesis

The use of plant supplements to compliment the health needs of consumers has gained popularity due to the confidence that consumers have in these products regarding their efficacy and assumed 'safety' even though there are no scientific proofs. However, in some instances, these products present some risks especially when they are adulterated or contaminated with plant constituents of concern. The aim of this thesis was to evaluate the potential presence of adulterants (i.e. PDE-5i) and contaminants such as PAs, PAHs, PCDD/Fs and dl-PCBs in herbal supplements sold for improving sexual performance in Accra (Ghana). Subsequently, an evaluation of potential health risks of consumers, upon exposure to these harmful constituents were assessed based on the estimated daily intakes (EDIs) in order to determine when risks management actions by relevant authorities was necessary.

This thesis is organized in six chapters, i.e. Chapter 1 (General introduction), Chapter 2 , 3, 4 and 5 (entails the main objectives of the thesis) and Chapter 6 (General discussion). **Chapter 1** presents a short background and description of the aims of the thesis. Terms such as **phosphodiesterase type-**

5 and its role in the smooth muscle cells was described. The constituents of potential concern (i.e. **adulterants**, **PAHs, dioxins and dl-PCBs, and PAs)** in the herbal supplements, their occurrence, potential adverse health effects, and how the risk upon exposure will be assessed were also discussed. The analytical methods employed in this thesis for the determination of the inhibition potentials of selected supplements (i.e. **PDE-Glo assay),** the presence of PDE-5i (**LC-MS)** and the AhR binding effects (**DR CALUX® assay**) of plant constituents in the supplements were also introduced in this chapter.

In Chapter 2, the supplements were screened to determine their inhibition potentials against PDE-5 enzyme activity. The screening outcomes aided in the identification of 'suspect samples' (i.e. those with estimated intakes above 100 mg sildenafil equivalents per day) which needed further investigations using hyphenated LC-MS procedures.

Chapter 3 was a follow-up on the results presented in chapter 2 where all 40 supplements were analyzed using LC-MS based methods in order to prove the accuracy of the bioassay and to identify potential adulterants (PDE-5i) added by producers to obtain the desired effect. Subsequently, one of the supplements which showed discrepancy between the results from the two methods applied was further investigated using LC-MS together with ^{1}H-NMR to elucidate the chemical structure of the unknown compound. This was to prove the advantages in combining biological assay and chemical analysis (a tiered approach) in the investigation of complex mixtures, and to demonstrate that high discrepancies between the two analysis was likely the cause of either a known or unknown compound(s) missed during the first chemical analytical MS analysis.

In **Chapter 4**, the potential presence of PAHs, dioxins and dl-PCBs possibly introduced into the finished products as a result of contaminated raw materials or during processing was investigated. Initially, supplements were screened using the DR CALUX® assay in order to identify the presence of stable (acid-silica clean-up and 48 hrs exposure) and less stable (without acid-silica clean-up and 4 hrs exposure) AhR-agonists. Ten supplements were subsequently selected based on screening outcomes for further analysis using gas chromatography mass spectrometry (GC-MS), in order to

conclude whether the observed DR CALUX® responses could be caused by known PAHs, dioxins and/or dl-PCBs.

In **Chapter 5**, the potential presence of PAs in the 40 supplements as a result of contaminated raw materials and/or the unintentional co-harvesting of raw materials with PA containing weeds, was examined using liquid chromatography mass spectrometry (LC-MS). The risk upon exposure to these contaminants was subsequently assessed.

In Chapter 3, 4 and 5, the concentration of each compound in the supplements was used to estimate the daily intakes and exposure levels of consumers. The risk of consumers were characterized based on available health-based guidance values or the MOE approach proposed by EFSA.

Finally, in **Chapter 6**, the main findings of the various chapters in this thesis were discussed. This chapter includes discussion on the results obtained, strengths and limitations of the methods applied, and the risk assessment approaches employed. Additionally, this chapter presents some future perspectives based on data gaps and limitations in performing risk assessment and the way forwards.

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Chapter 2

PDE-5 inhibitors in selected herbal supplements from the Ghanaian market for better erectile function as tested by a bioassay

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Abstract

Herbal supplements sold as 'all natural' on various markets in Accra (Ghana) and advertised as highly efficacious in treating erectile dysfunction (ED) were analyzed using a PDE-5 enzyme inhibition assay. The claimed efficacy of these products could be the result of inherent plant constituents, but also of intentionally added pharmaceuticals. Medically, ED is treated with potent inhibitors of the phosphodiesterase-5 (PDE-5) enzyme, as in the case of sildenafil. To test the inhibition potentials of the Ghanaian supplements, extracts were made and tested using a PDE-Glo phosphodiesterase assay, a luminescent high-throughput screening (HTS) method.

Results revealed that about 90% of the selected samples were able to inhibit PDE-5 enzyme activity at various degrees. Estimated concentrations in sildenafil equivalents ranged from traces to very high, with 25 samples (62.5%) pointing at daily doses higher than 25 mg sildenafil equivalents and 9 (22.5%) of these at doses higher than the maximum recommended daily intake of 100 mg sildenafil equivalents. Further investigations are needed to confirm if the observed effects are due to inherent plant constituents or merely the result of added synthetic PDE-5 enzyme inhibitors, especially because doses above 100 mg sildenafil equivalents per day may result in severe health risks.

2.1 Introduction

Stimulation of sexual drive is initiated in the brain, therefore activities that causes mental and emotional instability, including certain chronic medical conditions (diabetes, hypertension) may affect an individual's sexuality and overall sexual performance (Musicki et al. 2009). This may potentially result in a sexual dysfunction. Aging is another factor that contributes to the prevalence of sexual dysfunction, and this varies significantly between males and females (Laumann et al. 1999; Rosen 2000; Heiman 2002; Burnett, 2006; Lewis et al. 2010). Although dysfunction in sexual desire is less prevalent in men, erectile dysfunction (ED), which is the inability of a man to achieve and maintain an erection for satisfactory sexual performance has gained lots of attention due to its prevalence rate in the past two decades. Solutions ranging from psychological therapies to prescriptive drugs have been approved by health professionals for the treatment and management of ED since the early 1990s (Patel et al. 2014).

Currently, there are seven drugs approved for the treatment of ED. These include sildenafil citrate, sold as Viagra; tadalafil, sold as Cialis; vardenafil hydrochloride, sold as Levitra; udenafil, sold as Zydena; mirodenafil hydrochloride, sold as Mvix; iodenafil carbonate, sold as Helleva and avanafil, sold as Stendra (Patel et al. 2014). These drugs inhibit PDE-5 enzyme activity in the corpus cavernosum (smooth muscle cell), resulting in the accumulation of cyclic guanosine monophosphate (cGMP) to levels needed for the relaxation of the smooth-muscle cells thus increasing blood flow to the penis, and subsequently enhancing erection (Venhuis et al. 2008).

Different studies have also reported the PDE-5 inhibition potentials of certain plants and plant constituents, such as *Ginkgo biloba*, maca, red ginseng, and icariin (Shamloul 2010; Singh et al. 2012; Kotta et al. 2013; Ongwisespaiboon and Jiraungkoorskul 2017). These plants may be used individually or combined with other plants to potentiate the expected outcomes. Although they are often used in the raw state, others are (semi-)processed into powders, liquids or into finished products such as capsules, gels and ointment. Whilst some of these supplements are imported from other countries (i.e. China, Canada and the USA), the majority are locally produced and advertised

with attractive names such as, Be4be4, Touch Me and See, Hapimaan, Recharger, Alive Power, Bigger Longer More and Bazouka AK-47. The surge in market demand has resulted in their abundance on the open market with little to no attention paid to the quality or safety of end-products, several of which are sold illegally.

In most African countries including Ghana, individuals experiencing ED fail to consult with qualified professionals to address their sexual related matters due to the high consultation charges, social stigma and possible embarrassment they may encounter. They resort to self-medication by employing available remedies from various outlets. The herbal supplements sold for treatment or management of ED are much preferred due to the general perception of safety attached to natural products, to prevent the well-known adverse effects of synthetic PDE-5i, and to avoid possible drug-drug interactions (Lowe and Costabile, 2012; Ventimiglia et al. 2016). In addition, these herbal supplements have competitive selling advantage because they are relatively cheaper compared to the synthetic alternatives.

The use of herbal supplements for treating/managing ED is not only limited to individuals experiencing ED. Healthy men who have no issues regarding erectile function also use these products for recreational purposes, either to increase their penis sizes, boost their sexual desires or extend their performance time during intercourse (Harte and Meston 2011; Danquah et al. 2011). Various concerns have been raised by relevant stakeholders regarding the safety of these supplements as well as the effect on the health of consumers.

The purpose of this study was to screen selected herbal supplements to determine their PDE-5 inhibition potentials in order to assess their safety. To achieve this, the PDE-Glo phosphodiesterase bioassay, a luminescent high-throughput (HT) screening method, which runs on the principle of competitive inhibition of enzyme-substrate (PDE-5-cGMP) complexes, was employed. The assay is based on the principle that an inhibitor reduces the degradation of cGMP by PDE-5 enzyme thus promoting the accumulation of cGMP. More cGMP means more ATP will be expended in the phosphorylation process of protein kinase A (PKA) substrate, thus resulting in less ATP after the

phosphorylation process. The amount of ATP remaining determines the amount of light produced. Therefore, the amount of light produced directly correlates to the PDE-5 enzyme activity and inversely related to the concentration of inhibitor, (i.e. more inhibitor results in less light). Application of this bioassay enabled estimation of the levels of PDE-5 inhibitors in the supplements (expressed in sildenafil equivalents), enabling subsequent assessment of exposure and potential risks.

2.2 Materials and methods

2.2.1 Sample selection

Forty herbal supplements were collected based on pricing, popularity among users and recommendations by sellers. The majority of the samples were capsules (*n = 30*) and a few were powders (*n = 2*) and liquids *(n = 8)*. Samples were kept in their original packaging at room temperature. Prior to analysis, each sample was assigned a sample identity (ID). Table S1 shows details of the products (sample ID, origin, product form, and instructions for use). Hereafter, products are referred to by their allocated ID.

2.2.2 Chemicals and reagents

Sildenafil citrate (CAS 171599–83-0) was purchased from Carbosynth (UK), acetonitrile (CAS 75–05-8) and methanol (CAS 67–56-1) from Biosolve Chemie SARL (Valkenswaard, The Netherlands) and dimethyl sulfoxide (CAS 67–68-5) was from Merck (Darmstadt, Germany). Water was prepared using a Milli-Q water purification system. The PDE-Glo phosphodiesterase assay kit (Promega, CAT No. V1361) was purchased from Fisher Scientific (Madison, WI, USA). The kit contained 5× PDE-Glo Reaction buffer, 5× PDE-Glo Detection buffer, Protein Kinase A, 5× PDE-Glo Termination buffer, 1 mM cGMP solution, 1 mM cAMP solution, Kinase-Glo substrate and Kinase-Glo buffer. Phosphodiesterase 5A1 human recombinant (CAT No. E9034) and 3-isobutyl-1-methylxanthine (IBMX) (CAT No. I5879) were purchased from Sigma-Aldrich (Saint Louis, USA). Coaster 96-well, flat bottom, non-treated, non-sterile white polystyrene assay plates were purchased from Corning (NY, USA).

2.2.3. Sample pre-treatment

Half the capsuled or powdered samples were emptied into clean beakers and mixed, whereas liquid samples were manually agitated for 1 min. Thereafter, 100 mg of the solid samples or 100 µL of liquid samples were aliquoted into 2.5 mL polypropylene vails with caps and 1 mL extraction solvent ACN/H_2O (80/20, *v*/v) was added to each sample. As controls, 100 mg of a known positive sample containing icariin (P) and of a negative sample (N) were treated in the same way as the

samples, but both in duplicate, where one was spiked with 50 µL sildenafil stock solution (2 mg/mL = 4.2 mM). In addition, 1 mL ACN/H_2O (80/20, v/v) in duplicate was used as a chemical blank, where one was again spiked with 50 µL sildenafil stock solution. To ensure that all samples were of equal volume, 50 µL ACN/H_2O (80/20, v/v) was added to each sample that was not spiked with sildenafil. Next, mixtures were vortexed (Vortex-2 Gene) at speed 5 for one min then placed on a multi-tube vortex mixer (Heidoph Reax 2) for 30 min. Finally, samples were centrifuged (Eppendorf Centrifuge 5415 R) (985 *g*) for 5 min at 22°C. Supernatants were collected and transferred into new vials. A 100 µL portion of the supernatant was aliquoted into a vial already containing 100 µL DMSO (as a keeper) and mixed. The ACN/H_2O phase was then evaporated under continuous stream of nitrogen at 60°C for 40 min. Samples were further diluted, i.e. 10-fold, 100-fold, 1000-fold and 10,000-fold with DMSO when necessary.

2.2.4 PDE-5 enzyme inhibition assay

The analysis was performed following the protocol by PROMEGA (PDE-Glo™ Phosphodiesterase Assay Technical Bulletin). Initially, a system suitability test was performed as recommended by the manufacturer to demonstrate the specificity of the enzyme assay. To this end a sildenafil concentration response curve at concentrations, 0 nM (DMSO), 0.5 nM, 50 nM, 500 nM and 50,000 nM, was tested to determine the level of inhibition at the various concentrations. Additionally, a chemical blank containing all reagents (minus sildenafil), a spiked chemical blank (plus sildenafil), a positive control (P) (sample containing icariin), and a botanical preparation previously tested as negative in the PDE-5 assay was used as controls. The equation ($y = a0 + a1*\exp.(-x/a2)$ generated from the sildenafil dose response curve was used to fit the data.

2.2.5 Analysis of samples using the PDE-Glo assay

An aliquot of 5 µL sample extract, 7.5 µL PDE-5 enzyme and 12 µL (20 µM) cGMP were pipetted into a Coaster 96-well plate, mixed for 5 min on a plate shaker and incubated in the dark for 90 min. The process was terminated by adding 12 µL termination solution (termination buffer +100 mM 3-isobutyl-1-methylxanthine). Next, 12 µL detection solution (detection buffer + protein kinase A)

was added, mixed for 5 min and incubated for 20 min. Finally, 50 µL kinase glo reagent (kinase glo substrate + kinase glo buffer) was added and the plates were incubated for another 10 min. Luminescence signals (RLU) were measured using a Biotek Synergy HT (Vermont, USA). A schematic diagram of the assay protocol is presented in Fig. 1. Data analysis was performed with Microsoft Excel version 2016 and SlideWrite™ plus Version 6, and graphs were plotted using Microsoft Excel and GraphPad Prism 5.

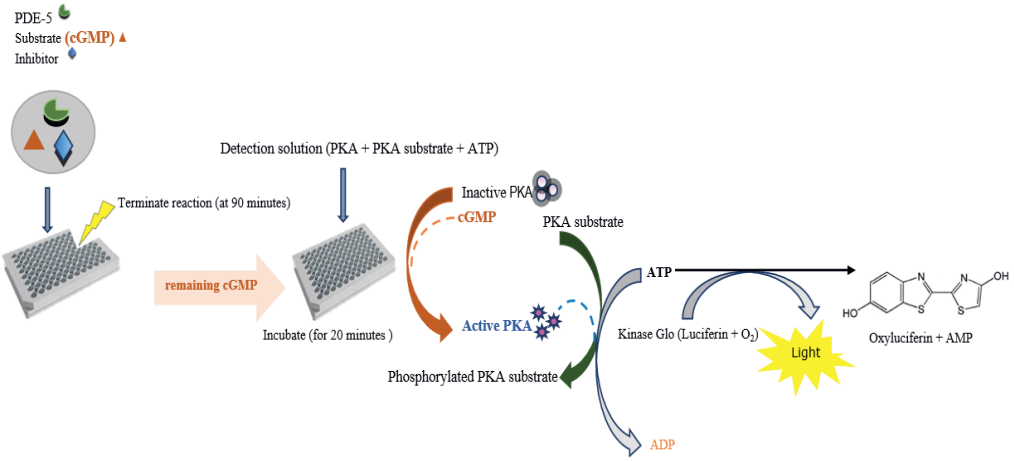

Figure 1. Schematic diagram of the PDE-Glo Phosphodiesterase assay: a PDE-5 inhibitor reduces PDE-5 enzyme activity. Remaining ATP is measured as emitted light which directly correlates to enzyme activity and is inversely related to the concentration of inhibitor.

2.2.6 Precision and repeatability

In order to test the reliability and repeatability of the assay protocol, three independent analyses were carried out on a selected number of samples (both liquid and capsules) four weeks after the initial analysis and one year after. Sample pre-treatment and analysis followed the same procedure as described above. The coefficient of variation (CV) from these three independent replicates was used to determine the levels of precision. The defined acceptance criterium was CV $\leq$ 20% among replicates.

2.2.7 Effects of matrix in sample extracts

It was noticed that majority of the undiluted sample extracts showed false positive and false negative screening outcomes in the PDE-5 enzyme inhibition assay due to matrix effects. Also during optimization of the assay protocol, matrix effect played significant role especially for undiluted solid sample extracts. This could be the result of interferences between analytes of interest and other substances present in sample extracts or the interaction of matrix constituents with enzymes or cofactors that form the bioassay, thereby influencing the measured light intensities. However, the effects of matrix reduced when sample extracts were further diluted.

2.3 Results and discussion

The purpose of this study was to determine the PDE-5 inhibition potentials of the selected supplements in order to assess their safety. To achieve this, samples were tested by the PDE-Glo bioassay followed by an estimation of the levels of PDE-5 inhibition expressed in sildenafil equivalents. Fig. 2 shows the concentration-response curve for sildenafil as obtained in the PDE-5 enzyme inhibition assay. Sildenafil results in a concentration dependent decrease in PDE-5 activity at a range of 0.5 to 50,000 nM. After mathematical curve fitting, an IC_{50} of 332 nM was calculated. It is important to stress that quantification of sildenafil equivalents in sample extracts showing responses in the range of the high and low plateau of the sildenafil standard curve will not be accurate. In these parts it can only be expressed as less than (left side) or greater than (right side) a certain concentration. A more precise quantification of the response can only be performed in the steep part of the curve (roughly the part of the curve between 50 and 500 nM sildenafil).

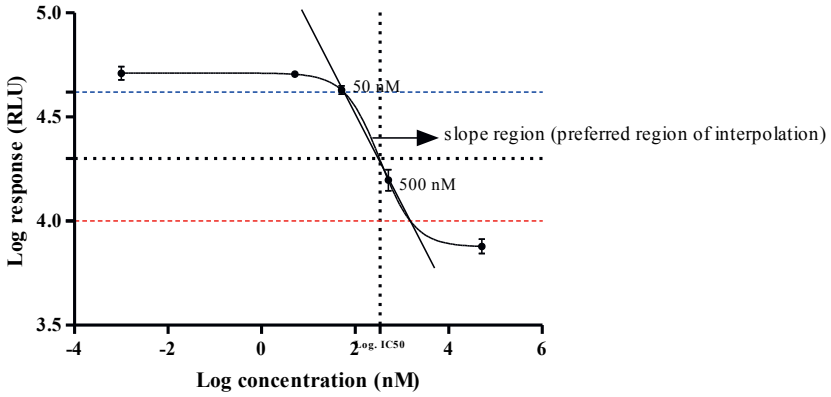

Figure 2. Sildenafil-concentration dependent reduction in PDE-5 activity. An IC_{50} of 332 nM was calculated from the fitted dose-response curve. Response expressed as luminescent signal (RLUs mean ± SD). The blue (above) and red (below) dotted horizontal lines indicate the RLU boundaries above and below which quantification is not possible.

To determine whether the assay was fit for purpose (i.e. to test herbal extracts), a chemical blank, a known positive sample (containing icariin) and a negative herbal sample (all three with and without a sildenafil spike were tested in two independent studies (Fig. 3). As expected, the unspiked chemical blank did not result in any inhibition of the PDE-5 activity while the spiked chemical blank showed an inhibition. The negative control sample showed a slight inhibition when tested undiluted, which was a false positive screening outcome. However, no clear inhibition was observed with diluted extracts of the negative control sample. When spiked with sildenafil at a rather low level of 1 mg/g, a clear decrease in PDE-5 activity was observed in all dilutions. The positive control sample showed a clear inhibition at 10-fold dilution, while the undiluted sample extract did not. This was ascribed to matrix effects, resulting in a false negative screening outcome when testing the undiluted sample extract. These outcomes suggest that undiluted sample extracts may result in both false negative and false positive screening outcomes and that testing serial dilutions of sample extracts is essential to screen for the presence or absence of PDE-5 inhibitors. As indicated, inhibition of PDE-5 activity by the spiked control samples decreased upon dilution, except for the 10,000-fold diluted extract of the spiked chemical blank. Using a fitted sildenafil curve and the amount spiked (1 mg/g), a recovery of 280% was calculated from the 100-fold diluted spiked chemical blank and 40% from the 100-fold diluted spiked negative control (based on the steep part of the sildenafil curve).

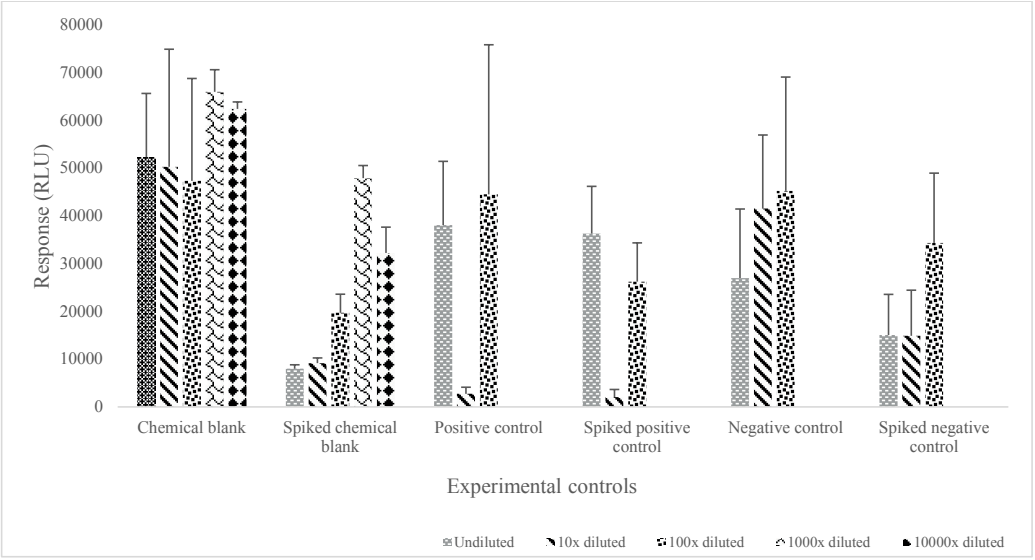

Figure 3. PDE-Glo phosphodiesterase assay responses obtained for experimental controls with and without a sildenafil spike (1 mg/g). Response expressed as luminescent signal in relative light units (RLUs) (mean ± SD; for two independent clean-ups).

Fig. 4 further demonstrates the matrix effect observed during analysis by showing the results from 6 sample extracts (i.e. 3 solid samples (powders) and 3 liquid samples). The extracts from solid samples were tested in higher dilutions based on the results from a pilot study. Matrix effects were observed for concentrated extracts from solid samples (i.e. undiluted and 10-fold diluted extracts of powdered samples), thus responses obtained from these dilutions were less reliable. As a result, the 100-fold diluted extracts of these solid samples show the highest inhibition. Further dilution resulted in less inhibition, as compound(s) responsible for the observed inhibition were overly diluted and the measured responses (RLUs) increased, thereby shifting response to the negative side of the curve. The matrix effect from these solid samples would have resulted in a false negative screening outcome if these three solid samples were tested undiluted. No clear matrix effects were observed when testing extracts of liquid samples. It was observed that further dilution only resulted in less inhibition and an increase in response (with the likelihood of shifting response to the negative side of the curve).

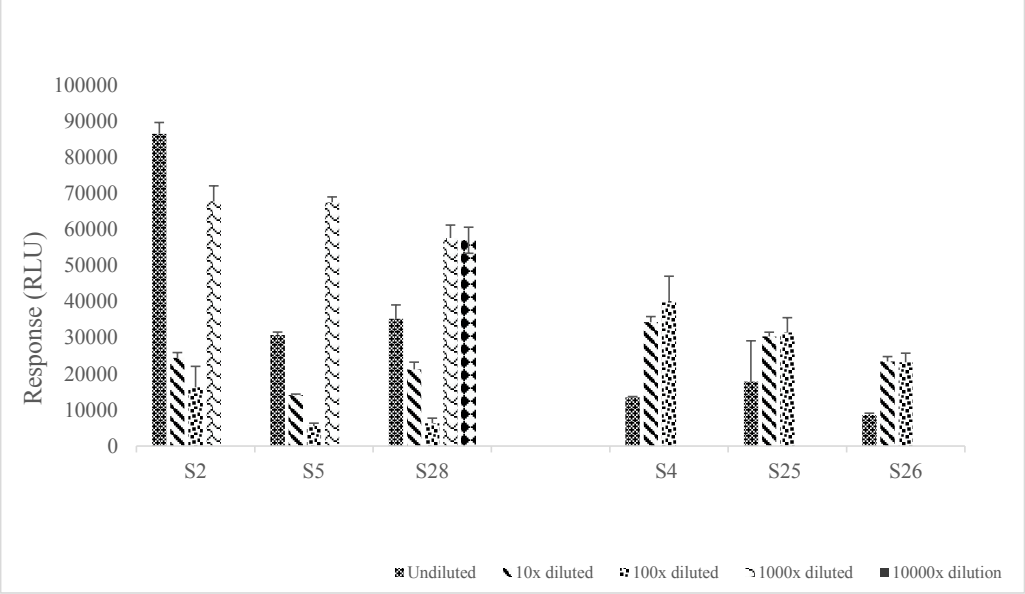

Fig. 4. PDE-Glo phosphodiesterase assay responses obtained with various dilutions from extracts of three solid (powdered) (S2, S5 and S28) and three liquid samples (S4, S25 and S26). Response expressed as luminescent signal in relative light units (RLUs mean ± SD).

Fig. 5 shows the response obtained from the 100- and/or 1000-fold diluted extracts of the 40 supplements. Initially, all sample extracts were tested from undiluted, 10- and 100-fold dilution. Based on the outcomes, most sample extracts were tested again at 1000- and even 10 000-fold dilution for same extracts. Overall, 36 (90%) out of the 40 samples showed the ability to inhibit PDE-5 enzyme activity at 100- and 1000-fold dilutions. The complete results obtained from the applied dilutions of sample extracts is presented in Fig. S1. The horizontal dotted lines in Fig. 5 indicate the steep part of the sildenafil calibration curve (Fig. 2) reflecting the range where conversion of the response into sildenafil equivalents is feasible. It became evident that the undiluted and 10-fold diluted extracts were not suitable for the conversion of responses (RLUs) to sildenafil equivalent amounts in samples (due to matrix effects as discussed above). One must also be aware that quantitative results obtained with the 10 000-fold diluted extracts were less accurate, as a magnification of potential errors may occur.

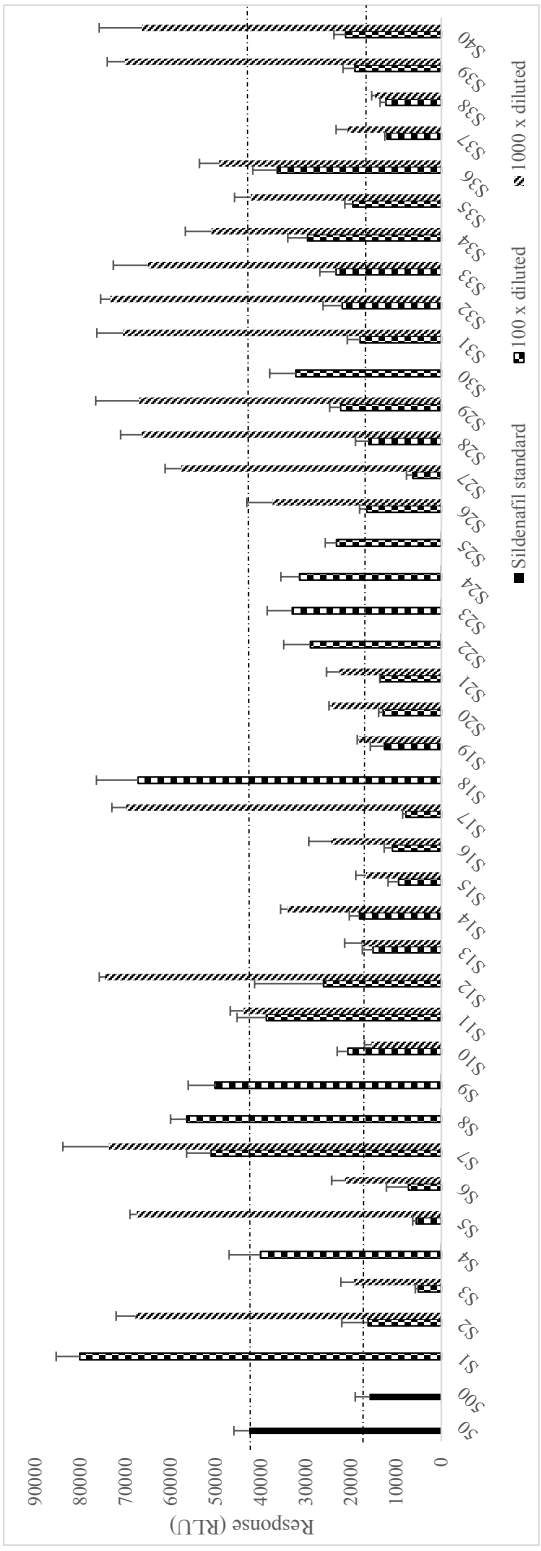

Fig. 5. PDE-Glo phosphodiesterase assay response (RLU) of the 40 extracts tested in 100- and 1000-fold dilutions. Based on the concentration response curve for sildenafil, responses between 16,000 RLUs (dotted line below) and 43,000 RLUs (dotted line above) can be converted to amounts in sildenafil equivalents. Response in RLUs expressed as mean ± SD for 3 measurements of each dilution.

Sample responses between 16,000 and 43,000 RLUs (the steep part of the sildenafil dose-response curve), were used to calculate the concentration in mg sildenafil equivalents per g of sample (Table S2). However, responses below 16,000 RLUs and above 43,000 RLUs, were not used for the calculations and were represented by less than (i.e. <0.07 and < 0.66 mg/g at 100- and 1000-fold dilutions, respectively) and greater than (i.e. > 8.2 mg/g at 100-fold dilution) the respective values. Table 1 shows the calculated sample concentrations based on the responses from the 100- and 1000-fold dilutions. Four supplements (i.e. S1, S7, S8, S18) showed high RLUs (i.e. no inhibition) for both the undiluted and diluted extracts, implying that no PDE-5 inhibitors were present in these samples. The other 36 samples showed a clear inhibition (i.e. low response) for the undiluted sample extracts which then often decreased (higher response) to some extent in higher dilutions. However, S3, S16, S19 and S20 still showed a near maximal inhibition at the higher dilutions, indicating the presence of high concentrations of PDE-5 inhibitors.

Table 1. Estimated concentrations and daily intakes (EDI) of PDE-5 inhibitors in 40 supplements based on dilutions that resulted in an inhibition response in the steep part of the sildenafil calibration curve. Levels for the other dilutions are expressed as lower or higher than based on the boundaries of the steep part.

Sample ID	Concentration (mg sildenafil-equivalents per gram)		Average weight of dose unit (g or mL)	No. of dose units per day	Estimated daily intake (mg per day)		Evaluation
	100-fold [a]	1000-fold [a]			100-fold [a]	1000-fold [a]	
S1	< 0.07		1.07	4	< 0.3		N
S2	4	< 0.66	0.81	4	14	< 2	L
S3	> 8.2	36	1.01	2	> 17	72	M
S4 (l)	0.8		30	3	71		M
S5	> 8.2	< 0.66	1.01	4	> 36	< 3	M
S6	> 8.2	31	0.47	2	> 8	29	M
S7(l)	< 0.07	< 0.66	30	4	< 8	< 79	M
S8	< 0.07		2.01	6	< 1		N
S9	< 0.07		0.51	2	< 0.1		N
S10	3	47	0.46	2	3	43	M
S11	1	5	0.49	2	1	5	L
S12	2	< 0.66	0.74	2	3	< 1	L
S13	5	40	1.31	4	25	208	H
S14	4	1	1.16	4	18	62	M
S15	> 8.2	43	0.36	6	> 18	92	M
S16	7	26	0.65	2	9	34	M
S17	> 8.2	< 0.66	1.1	4	> 36	< 3	M
S18	< 0.07		0.65	4	< 0.2		N
S19	6	39	0.43	2	5	33	M

S20 (l)	6	26	10	3	173	782	H
S21	6	29	0.97	4	21	112	H
S22 (l)	2		30	3	172		H
S23 (l)	1		20	1	29		M
S24 (l)	2		9	2	29		M
S25 (l)	3		60	1	167		H
S26	4	10	1.1	2	10	22	L
S27	> 8.2	< 0.66	1.67	4	> 55	< 4	M
S28	5	< 0.66	2.13	6	57	< 8	M
S29	3	< 0.66	1.09	4	13	< 3	L
S30	2		30	4	185		H
S31	4	< 0.66	1.35	4	21	< 4	L
S32	3	< 0.66	1.24	4	15	< 3	L
S33	3	< 0.66	1.53	4	17	< 4	L
S34	2	< 0.66	1.34	4	10	< 4	L
S35	4	6	15	4	210	368	H
S36 (l)	1	1	0.96	2	2	2	L
S37	6	32	2.03	4	51	263	H
S38	6	50	1.47	4	36	292	H
S39	4	< 0.66	1.7	4	25	< 5	M
S40	4	< 0.66	1.14	2	7	< 2	L

[a] calculated from the response obtained with either the 100-fold or 1000-fold diluted sample extract

(l) : represents liquid samples

Evaluation : N: negative, L: < 25 mg/d; M: 25-100 mg/d; H: >100 mg/d

In a number of cases (e.g. S15), the response for the undiluted extract was high (implying a low concentration of inhibitors), but responses started to decrease at higher dilutions, showing that these samples do contain a substantial amount of PDE-5 inhibitors. For these samples the previously described matrix effect with undiluted sample extracts would actually have resulted in a false negative screening outcome when using the result from the undiluted and in some cases 10-fold diluted extracts.

It is evident from Table 1 (Table S2) that the levels of inhibition in the supplements differed for each dilution (especially due to matrix effects in the concentrated extracts of solid samples), nonetheless the assay clearly identified samples which contained high levels of PDE-5 inhibitors. For the majority of the samples, the different dilutions gave a response within the range of the calibration curve enabling estimation of the sample concentration in sildenafil equivalents. These outcomes were eventually used to classify supplements as being negative (N), low (L), medium (M) or of high (H) concentration.

Next, the average weight of the recommended daily dose of each supplement was multiplied by the estimated concentrations expressed in sildenafil equivalents (mg/g) to derive the intakes per day (expressed in mg sildenafil equivalents per day) (Table 1). A complete list of the estimated intakes of all dilutions is presented in Table S3. The daily use of the supplements based on the recommendation on the label and the resulting daily intakes ranged from traces to levels above 100 mg sildenafil equivalents per day. Based on the 100- and 1000-fold dilutions (most relevant dilutions), 4 (10%) out of the 40 samples were found to be negative in inhibiting PDE-5 activity whereas 36 (90%) resulted in detectable intake levels of PDE-5 inhibitors. Out of the 36 positive samples, 11 (31%) resulted in a relatively low daily intake of less than 25 mg sildenafil equivalents, 16 (44%) resulted in daily intakes between 25 and 100 mg, and 9 (25%) resulted in intakes above 100 mg sildenafil equivalents (Table 1).

According to Pfizer, sildenafil citrate (Viagra) is available in three dosages, i.e. 25, 50 and 100 mg, of which the recommended dosage for an average patient is usually 50 mg. Based on the individual's

circumstances and severity of ED, the recommended dosage may be down-scaled to 25 mg or up-scaled to 100 mg once per day [2]. Comparing the estimated amounts taken when using the supplements to the recommended daily dosage to Viagra (25–100 mg), it was assumed that supplements resulting in daily doses below 25 mg may not have the desired effect and therefore will not potentially pose a risk to consumers. Also, samples resulting in daily doses of 25 to 100 mg sildenafil equivalents may cause an effect but may not likely result in fatalities because they were within the recommended dose range. Nonetheless, individual differences may play some roles with regards to bioavailability, as a result, concentrations from 25 to 100 mg may be of concern to health authorities. However, supplements with concentrations above 100 mg sildenafil equivalents may be considered of high risk especially to vulnerable individuals. The high amounts of sildenafil (above 100 mg) may cause extreme relaxation of the smooth muscle cells which can inadvertently put the lives of unsuspecting consumers at unanticipated risks such as ischemic (low-flow) priapism which may lead to penile fibrosis of the corpus cavernosa and instead result in ED (Broderick et al. 2010; Zheng et al., 2013). There is also a risk for individuals using antihypertensive drugs such as organic nitrates (e.g. nitroglycerine, doxazosin and terazosin) (Boden et al., 2012) and patients suffering from hypotension (Kloner, 2007) for whom the use of PDE-5 inhibitors is contraindicated due to possible drug-drug interactions.

[2] Getting Started with Viagra (Sildenafil citrate). https://www.viagra.com/taking/finding-the-right-dose (accessed on 17th July 2020).

2.4 Conclusions

This study revealed the inhibition potentials of herbal supplements sold on markets and pharmacies in Accra (Ghana) for improving sexual performance. Out of the 40 selected samples, 36 (90%) were able to inhibit PDE-5 enzyme activity at various degrees. Twenty-five (62.5%) out of the 36 showed a response that implied a daily dose of over 25 mg sildenafil equivalents and 9 (22.5%) out of the 36 would result in a daily dose above 100 mg sildenafil equivalents, which is higher than the highest recommended daily dose of Viagra for treating ED in patients.

The PDE-Glo phosphodiesterase assay proved its suitability as a screening tool for the selection of 'suspect samples' that required further investigations by LC-MS to disclose the identity of the compound(s) eliciting the observed inhibition of PDE-5 enzyme activity. These compounds may be natural plant constituents like icariin (as in the case of the positive control), but also illegally added synthetic PDE-5 inhibitors and or their analogues (Singh et al. 2009; Patel et al. 2014; Rocha et al. 2016). In case of unknowns, subsequent analysis based on bioassay-guided fractionation combined with LC-(HR) MS/MS analysis could lead to the discovery of the active compounds responsible for the observed responses in the PDE-5 enzyme inhibition assay.

Collaboration

This project is a collaboration between the Dept. of Toxicology and Wageningen Food Safety Research at the Wageningen University and Research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY TABLES

***Table S1:*List of samples (ID), origin, forms, ingredients, dosage and health claims**

Sample ID	Ingredients	Dosage	Health claims
S1[c,a]	❖ Mondia whitei ❖ Tribulus terrestris ❖ Puerana tuberos ❖ Dioscorea bulbifera	Two capsules, twice daily after meals for four week or until you have the desire results	❖ Boosting erectile functions in men ❖ Prolong libido ❖ Eliminate waist pains ❖ Eliminate frigidity in both men and women
S2[c,a]	❖ Cryleepis Sanguinelenta preservative	Take two capsules twice daily after meals	❖ For sexual weakness ❖ Waist pains ❖ Increase power of resistance ❖ Produce sufficient physical energy ❖ For delay release of sperm ❖ Joint pains gives an extra strength
S3[c,a]	❖ Tribulus terrestris ❖ Roxb ❖ Ginkgo bilaba ❖ Cayenne panex caltrop	Take two capsules thirty minutes before sexual intercourse	❖ Rejuvenation and vitality
S4[l,a]	❖ Coconut Roots ❖ Kraman kote ❖ Tiger knot	3 tots daily	❖ Waist pains ❖ Sexual weakness ❖ Reduce Phlegm
S5[p,a]	❖ Maca root ❖ Ginseng ❖ Black pepper ❖ Cinnamon ❖ Ginger ❖ Garlic ❖ Tumeric	Mix one table spoonful of this powder with any alcoholic beverages	❖ Improve energy, vitality and sexual performance ❖ Support of the male glandular and hormone system
S6[c,a]	❖ Penianthus Zenkeri ❖ Clausena anisata	Two capsules with meals 30 minutes before sex	❖ For male sexual weakness, stamina and low sperm counts

S7[l,a]	❖ N/A	N/A	❖ Erectile malfunction ❖ Early ejaculation ❖ Low libido
S8[c,a]	❖ Magnesium Steafe-20mg ❖ Euadenia eminens-80mg ❖ Mondia whitei-100mg ❖ Phenocentrum jollyanum-30mg ❖ Paullinia pinnata-40mg	Two capsules three times daily or three capsules two times daily	❖ For loss of appetite ❖ Restores energy ❖ Enhances libido
S9[c,a]	❖ N/A	N/A	❖ Improve sexual function fundamentally
S10[c,b]	❖ N/A	N/A	❖ Safe and natural approach to enlarge the penis ❖ Better duration and better stiffness ❖ Easier control of ejaculation ❖ Greater healthy sexual euphotial
S11[c,d]	❖ N/A	Take 2 capsules everyday	❖ For good test and sex excitements
S12[c,a]	❖ Paullinia-Pinnata ❖ Abrus-Precatorius ❖ Daucus Carota	One capsule two times daily after meal	❖ Waist men ❖ Premature ejaculation
S13[c,a]	❖ Cissus populnea ❖ Cyperus esculentus ❖ Musa paradisiaca ❖ Epimedium grandifilorum/Horny goat weed extract	Two capsules, two times daily after meal	❖ Male vitality
S14[c,a]	❖ Lepidium Meyetini ❖ Turnera Diffuse ❖ Ptychopetalum ❖ Herbal extracts	Two capsules, twice daily after meals	❖ Good for erectile dysfunction (Sexual weakness) ❖ Premature ejaculation ❖ Retarded ejaculation ❖ Reduced libido and waist pains ❖ It boots sperm count, immune system and general well being

			❖
S15[c,a]	❖ N/A	N/A	❖ Good curative effect on a prostate sicker ❖ Promote man's genitalia growing up for the second time ❖ All patients of hypertension and heart disease can take the medicine
S16[c,b]	❖ N/A	Take two capsules 30 minutes before making love	❖ N/A
S17[c,a]	❖ Moringa oleifera ❖ Euadenia eminens ❖ Delbergia saxatilis ❖ Sphenocentrum jollyanum	Two capsules, two times daily	❖ Male vitality
S18[c,a]	❖ Paullinia pinnata ❖ Mondia whitei ❖ Khaya senegalensis ❖ Cyperus esculentus	Two capsules twice daily after meals	❖ Vigor and vitality
S19[c,b]	❖ N/A	N/A	❖ Impotence and ejaculation ❖ Make it big, thick, long lasting and increasing sperms
S20[l,b]	❖ Rehmannia root ❖ AC-hyranthes root ❖ Dodder seed ❖ Fruit of puncturevine ❖ Cnidium fruit ❖ Herb of Korean epimedium ❖ Fruit of magnoliavine ❖ Aconite root ❖ Red ginseng ❖ Astragalus root ❖ Rhodiola root	I bottle for each time, 1-2 times a day or two bottles 30 minutes before coition each time	❖ Promoting sexual function ❖ Enhancing the body's nature curing ability ❖ Adjusting and balancing the body's multi-sexual elements ❖ Boosting overall immunity. ❖ It is suitable for the symptoms caused by the low sexual vitality including male impotence, erectile dysfunction, lassitude of the waist and knees, chilly sensation and coldness of limbs, fatigue and so on
S21[c,a]	❖ Penianthus Zenkeri ❖ Clausena anisata	Two capsules twice daily after meals	❖ For male sexual weakness, stamina and vigor

S22[l,a]	❖ Xylopia aethiopica ❖ Khaya senegaiensis ❖ Paulina pinnata ❖ Rauwolfia vomitoria ❖ Psidium guajava	Two table spoonful (30ml) three times daily after meals	❖ Sexual weakness piled (Kooko) ❖ Low sperm count ❖ Waist pains
S23[l,a]	❖ Lavang 5% ❖ Sunthi(Zingiber officinate, ginger) 20% ❖ Pippali (piper lorgum) 20% ❖ Piper nigrum (Kali Mirch) Black pepper 15% ❖ Kapikaachhu seeds (Mucuna puriens) 40% ❖ Ashwagandha (Withania Somonifera)(Winter cherry) 50% ❖ Crocus sativus(Kesar) 4% ❖ Purified asphaltum (Shilajit) 75%	20ml before bed	❖ Helps to cure impotence ❖ Helps to cure premature ejaculation ❖ Increases stamina and reduces sexual fatigue ❖ Cures dysfunction ❖ Increases drive and libido ❖ Makes you feel confidence ❖ Increases sperm count ❖ Increases long time lasting sex ❖ Increases stamina and sex drive ❖ Increase Maximum sexual pleaser ❖ Increase Blood flow to the genitals ❖ Increase penis size and rock hard erection ❖ Increase control of ejaculation
S24[l,a]	❖ Paulling ❖ Pinnate ❖ Kyaya Senegalensie ❖ Piper guinease	1 tot 2x daily (morning and evening)	❖ Kooko (Piles) ❖ Constipation ❖ Waist pains ❖ Release of phlegms ❖ Sexsual weakness
S25[l,a]	❖ N/A	¼ glass cup two hours before having sex	❖ Makes a man erect and prevent frequent ejaculation
S26[c,a]	❖ N/A	2 capsules twice daily before meals.	❖ Pile, constipation, fever, waist pains and virility in men.
S27[c,a]	❖ Radix ginseng 100% ❖ Ginkgo biloba Kotu cola, daminana	2 capsules twice daily, after meals.	❖ For vitality
S28[c,a]	❖ Kigelia Africana ❖ Anthocleista nobilis ❖ Afromomum melegueta ❖ Turraea heterophylla	2 capsules three times daily before meals.	❖ For gonorrhea, syphilis ❖ Difficult in urinating ❖ Waist pains ❖ Candidiasis (white)

S29[c,a]	❖ Paullinia pinnata	2 capsules twice daily after meals	❖ Male vitality
S30[c,a]	❖ N/A	2 capsules twice daily after meals	❖ Waist pains ❖ Sexual weakness ❖ Joint pain ❖ Low sperm count
S31[c,a]	❖ Khaya ivorensis ❖ Paullinia pinnata ❖ Capparis erythrocarpos Clausena anisata	Take two capsules two times daily (Morning and evening)	❖ Male vitality
S32[c,a]	❖ Alstonia boonei ❖ Rauvolfia vomitoria ❖ Kyaya senegalensis Paullina pinnata ❖ Sphernnocentrum jollyanum pierre ❖ Cinnamon	Two capsules twice daily after meals.	❖ Virility ❖ Waist pains
S33[c,a]	❖ Radix ginsing 100% ❖ Ginkgo biloba ❖ Kotu cola ❖ Daminana	Take two capsules two times daily from 20 yrs and above only	❖ For sexual weakness ❖ Waist pain ❖ Mental stress ❖ Produces sufficient physical energy ❖ Release Siemens and give extra power
S34[c,a]	❖ Eudenia eminens ❖ Corynanthe pachyceras ❖ Paullinia pinnata ❖ Cyperus esculentus ❖ Spathodea campanulata	Take two capsules two times daily Take one hour before meals	❖ Waist pains ❖ General body pains
S35[l,a]	❖ N/A	4 tablespoons 3 times daily after meals	❖ Sexual weakness ❖ Waist pains ❖ Premature ejaculation
S36[c,a]	❖ Panax Ginseng ❖ Ginkgo biloba ❖ Damiana ❖ Hawthorn	1 Capsule Twice a day	❖ Improve erectile function ❖ Increase penis size

	❖ Horny Goat ❖ Catuaba ❖ Muira Puama ❖ Tribulus Cayenne fruit		
S37[c,a]	❖ Pauling Pinnata ❖ Sphenocentrum Jollyanum ❖ Tripiotaxi ❖ Eudinia	2 Capsules 2 times a day	❖ Sexual weakness ❖ Waist pain ❖ Pre-mature ejaculation
S38[c,a]	❖ Penianthus zenkeri ❖ Clausena anisata	2 Capsules 2 times a day	❖ Sexual vitality ❖ Waist pain
S39[c,a]	❖ Eudenia eminens ❖ Pisonia aculeata ❖ Paulina Pinnata ❖ Alchornea cordifolia ❖ Cocos nucifera ❖ Sida acuta ❖ Holiotropium indicum	2 Capsules 2 times a day	❖ Sexual weakness
S40[c,a]	❖ Paulina Pinnata ❖ Corynanthe ❖ Khaya ivorensis ❖ Rauwolfia vomitoria	2 Capsules a day	❖ Sexual weakness

Samples forms : [l] liquid [C]capsule [P]powder
Country of origin : [a] – Ghana [b] – China [d] – U.A.E. [e] – Canada
N/A : Information not available

Table S2: Estimated sample concentrations (nM) per each well in various dilutions as calculated from respective response (RLU), and final concentration in milligram (sildenafil equivalent).

Sample ID	(RLUs) mean ± SD					Amount (pg) per incubation (or 5 ul extract)					Conc. (mg sil equivalent per gram of sample)				
	Undiluted	**10x diluted**	**100x diluted**	**1000x diluted**	**10000x diluted**	**Undiluted**	**10x diluted**	**100x diluted**	**1000x diluted**	**10000x diluted**	**Undiluted**	**10x diluted**	**100x diluted**	**1000x diluted**	**10000x diluted**
CB	64096±3250	68167±3524	66781±3550	65983±4631	62405±1460	-3548	-4487	-4174	-3991	-3138	< 0	< 0.01	< 0.07	< 0.66	< 6.6
SCB	8264±1088	9145±1635	23065±2000	47910±2640	32245±5402	65618	47975	14044	1012	7683	> 0.08	> 0.82	2.8	2.0	154
PC	49971±3253	1789±369	73059±1762			337		-5534			0.001	> 0.82	< 0.07		
SPC	45054±3775	620±53	31813±3502			2006		7923			0.00	> 0.82	1.6		
NC	39822±5020	55274±2175	62186±7629			4044	-1256	-3084			0.01	< 0.01	< 0.07		
SNC	22670±2675	23412±3111	44992±656			14399	13740	2028			0.03	0.27	0.4		
S 1	28941±9829	74318±1247	80109±5261			14230	-5791	-6913			0.028	< 0.01	< 0.07		
S2	86534±3110	24406±1496	16268±5810	67775±4307		-8056	12905	22046	-4399		< 0	0.26	4.4	< 0.66	
S3	3179±547	3406±246	5139±629	19321±2948	13878±3613				17858	26584		> 0.82	> 8.2	35.7	532
S4 (l)	13472±234	34291±1580	40113±6942			27533	6599	3922			0.06	0.13	0.78		
S5	30725±853	14079±313	5554±789	67539±1479		8549	26138		-4346		0.02	0.52	> 8.2	< 0.66	
S6	38157±6569	7938±696	7307±4867	21391±2919	22051±281	4763			15617	14975	0.01	> 0.82	> 8.2	31.2	299
S7 (l)	59285±5473	53288±3801	51040±5415	68059±3688		-2347	-681	0	-5672		< 0	< 0.01	< 0.07	< 0.66	
S8	41433±3261	55090±8152	56462±3550			3382	-1204	-1589			0.01	< 0.01	< 0.07		
S9	6619±1022	36913±3938	50209±5897				5327	261			> 0.08	0.11	< 0.07		
S10	45566±556	23691±2090	20742±2339	15486±1549	18515±2415	1822	13500	16281	23369	18843	0.00	0.27	3.3	46.7	377
S11	66348±3588	45790±1945	38772±6513	43836±2944		-4075	1742	4493	2453		< 0	0.03	0.9	4.9	
S12	66934±7693	37224±5773	17350±2234	74560±1265		-4209	5184	11578	-5840		< 0	0.10	2.3	< 0.66	
S13	61472±6374	35378±2923	15176±2337	17748±3681	9601±261	-2906	6056	23932	19854	43670	< 0	0.12	4.8	39.7	> 816
S14	65724±4948	19584±3563	18125±2275	34099±1533		-3931	17552	19347	6697		< 0	0.35	3.9	13.4	
S15	43555±7081	10206±1399	9479±2349	16726±2228	16175±1486	2559	39504	44696	21329	22197	0.01	0.79	> 8.2	42.7	444
S16	6852±2197	8119±916	10875±1791	24417±4955			73603	36034	12896		> 0.08	> 0.82	7.2	25.8	
S17	35917±1869	13147±1060	7858±717	69888±3132		5796	28345		-4865		0.01	0.57	> 8.2	< 0.66	
S18	32280±3148	42344±6301	67253±9195			7663	3023	-4282			0.02	0.06	< 0.07		

S19	21369±644	11201±779	12640±3105	18189±458	20747±3621	15639	34622	29720	19264	16275	0.03	0.69	5.9	38.5	326
S20 (l)	7612±1592	8845±979	12943±951	24242±667			51821	28882	13039		> 0.08	> 0.82	5.8	26.1	
S21	53900±2486	40484±2175	13556±109	22679±2771		-861	3768	27331	14391		< 0	0.08	5.5	28.8	
S22 (l)	7529±1784	20293±1851	29058±5891				16759	9567			> 0.08	0.34	1.9		
S23 (l)	23186±6488	34858±8525	33060±5535			13937	6313	7241			0.03	0.13	1.4		
S24 (l)	22943±9977	30372±1176	31444±4125			19746	8758	8132			0.04	0.18	1.6		
S25 (l)	8602±498	23430±1363	23211±2520			56019	13724	13915			> 0.08	0.27	2.8		
S26	57090±2727	11250±641	16530±1589	37409±5714		-1761	34424	21631	5099		< 0	0.69	4.3	10.2	
S27	35224±3875	21238±2005	6315±1413	57635±3593	57015±3615	6132	15770		-1909	-1741	0.01	0.32	> 8.2	< 0.66	< 6.6
S28	55521±905	12058±842	16060±2967	66443±4644		-1326	31493	22386	-4097		< 0	0.63	4.5	< 0.66	
S29	34487±1721	36771±373	22332±2425	67003±9597		6500	5392	14710	-4225		0.01	0.11	2.9	< 0.66	
S30	63966±3143	11399±1494	32230±5817			-3517	33831	7691			< 0	0.68	1.5		
S31	62697±3047	53554±3607	18005±2838	70534±5830		-3210	-760	19507	-5004		< 0	< 0.01	3.9	< 0.66	
S32	71184±4992	12677±714	21972±4263	73371±2143		-5142	29613	15050	-5598		< 0	0.59	3.0	< 0.66	
S33	72901±1452	25553±3526	23359±3565	65057±7649		-5501	12002	13786	-3776		< 0	0.24	2.8	< 0.66	
S34	72215±4742	71276±4009	29687±4349	50963±5787		-5359	-5162	9174	25		< 0	< 0.01	1.8	< 0.66	
S35	33220±1097	12233±895	19622±1755	42242±3596		7155	30933	17509	3063		0.01	0.62	3.5	6.1	
S36 (l)	12320±5412	28085±3932	36360±5438	49250±4365		30664	10200	5585	569		0.06	0.20	1.1	1.1	
S37	67824±2646	18996±1391	12051±484	20845±2499	23220±1419	-4410	18247	31514	16173	13908	< 0	0.36	6.3	32.3	278
S38	82027±1351	31347±5623	12292±1301	14716±731	34739±5701	-7264	8188	30750	24813	6373	< 0	0.16	6.2	49.6	127
S39	55763±2135	28483±1483	19148±2641	70166±3890		864	9938	18064	-4925		0.00	0.20	3.6	< 0.66	
S40	16406±5433	13979±2035	21251±2572	66342±9498		21826	26358	15757	-4074		0.04	0.53	3.2	< 0.66	

KEY: CB - Chemical Blank ; SCB - Spiked Chemical Blank ; PC - Positive Control ; SPC - Spiked Positive Control; NC - Negative Control ; SNC - Spiked Negative Control

Note: Sample dilutions with a response above 43,000 RLU is represented by less than (i.e <0.07 and <0.66 mg/g at 100- and 1000-fold dilutions) whereas samples with response below 16,000 RLU is represented by greater than (i.e > 8.2 mg/g at 100-fold dilution).

Table S3: Estimated concentration and daily intake of unknown PDE-5 inhibitors in 40 supplements, expressed in mg sildenafil equivalents. Quantification was performed only for dilutions that resulted in a PDE-5 inhibition response in the steep part of the sildenafil calibration curve. The levels for the other dilutions are expressed as lower or higher than based on the boundaries of the steep part.

	Concentration (mg sildenafil-equivalents per gram)					Average weight of dose unit	No. of dose units	mg (intake per day)					
Sample ID	1[a]	10[a]	100[a]	1000[a]	10000[a]	(g or mL)	per day	1[a]	10[a]	100[a]	1000[a]	10000[a]	Evaluation
S1	0.03	< 0.01	< 0.07			1.07	4	0.1	<0.0	<0.3			N
S2		0.26	4.4	<0.66		0.81	4		1	14	<2		L
S3		> 0.82	> 8.2	35.7	532	1.01	2		>1.7	>17	72	1074	M
S4 (l)	0.06	0.13	0.78			30	3	5	12	71			M
S5	0.02	0.52	> 8.2	< 0.66		1.01	4		2	>36	<3		M
S6	0.01	> 0.82	> 8.2	31.2	299	0.47	2		>1	>8	29	282	M
S7 (l)		< 0.01	< 0.07	< 0.66		30	4		<1	<8	<79		M
S8	0.01	< 0.01	< 0.07			2.01	6		<0.1	<1			N
S9	> 0.08	0.11	< 0.07			0.51	2	>	<0.1	<0.1			N
S10		0.27	3.3	46.7	377	0.46	2	<	<0.2	3	43	347	M
S11		0.03	0.9	4.9		0.49	2		<0.1	1	5		L
S12		0.10	2.3	< 0.66		0.74	2		<0.2	3	<1		L
S13		0.12	4.8	39.7	>816	1.31	4		1	25	208	>4276	H
S14		0.35	3.9	13.4		1.16	4		2	18	62		M
S15	0.01	0.79	> 8.2	42.7	444	0.36	6		2	>18	92	959	M
S16	> 0.08	> 0.82	7.2	25.8		0.65	2	>	2	9	34		M
S17	0.01	0.57	> 8.2	< 0.66		1.1	4		2	>36	<3		M
S18	0.02	0.06	< 0.07			0.65	4		0.2	<0.2			N
S19	0.03	0.69	5.9	38.5	326	0.43	2		1	5	33	280	M

S20 (l)	> 0.08	> 0.82	5.8	26.1		10	3	>2	>31	173	782		H
S21		0.08	5.5	28.8		0.97	4		<0.3	21	112		H
S22 (l)	> 0.08	0.34	1.9			30	3	>7	30	172			H
S23 (l)	0.03	0.13	1.4			20	1	<0.6	3	29			M
S24 (l)	0.04	0.18	1.6			9	2	<0.7	3	29			M
S25 (l)	> 0.08	0.27	2.8			60	1	>5	17	167			H
S26		0.69	4.3	10.2		1.1	2		1	10	22		L
S27	0.01	0.32	> 8.2	< 0.66	<6.6	1.67	4	<	2	>55	<4	<44	M
S28		0.63	4.5	< 0.66		2.13	6		8	57	<8		M
S29	0.01	0.11	2.9	< 0.66		1.09	4	0.1	0.5	13	<3		L
S30		0.68	1.5			30	4		81	185			H
S31		< 0.01	3.9	< 0.66		1.35	4		<0.1	21	<4		L
S32		0.59	3.0	< 0.66		1.24	4		3	15	<3		L
S33		0.24	2.8	< 0.66		1.53	4		2	17	<4		L
S34		< 0.01	1.8	< 0.66		1.34	4		<0.1	10	<4		L
S35	0.01	0.62	3.5	6.1		15	4	1	37	210	368		H
S36 (l)	0.06	0.20	1.1	1.1		0.96	2	<0.1	<0.4	2	2		L
S37		0.36	6.3	32.3	278	2.03	4		3	51	263	2259	H
S38		0.16	6.2	49.6	127	1.47	4		1	36	292	749	H
S39	0.00	0.20	3.6	< 0.66		1.7	4		1	25	<5		M
S40	0.04	0.53	3.2	< 0.66		1.14	2	0.1	1	7	<2		L

1[a]: represents undiluted sample extract

[a]: represents dilution factor

(l) : represents liquid samples

Evaluation : N: negative, L : < 25 mg/d; M: 25-100 mg/d; H: >100 mg/d

SUPPLEMENTARY FIGURES

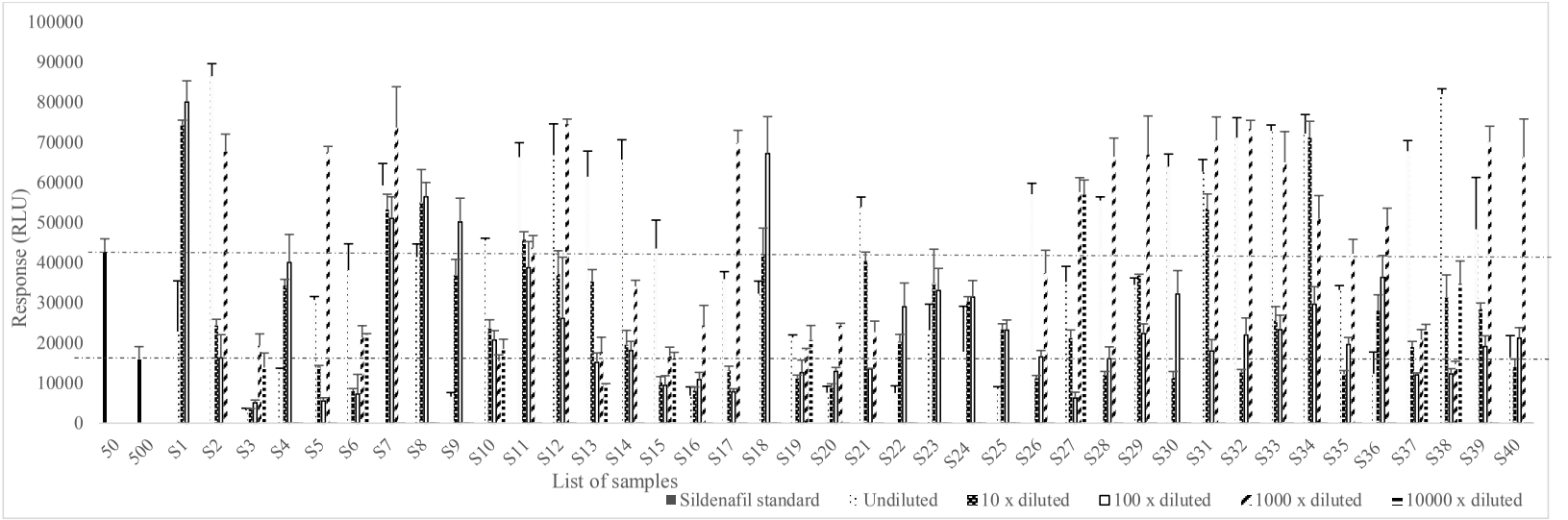

Figure S1. Responses from the 40 extracts in varying dilutions (i.e. undiluted, 10-,100-,1000- and 10000-fold) tested in the PDE-Glo phosphodiesterase assay. Based on the concentration response curve for sildenafil, responses between 16,000 RLUs (dotted line below) and 43,000 RLUs (dotted line above) was used in calculating the amounts of inhibition concentration in sildenafil equivalents. Response expressed as mean ± SD for measurements in triplicates.

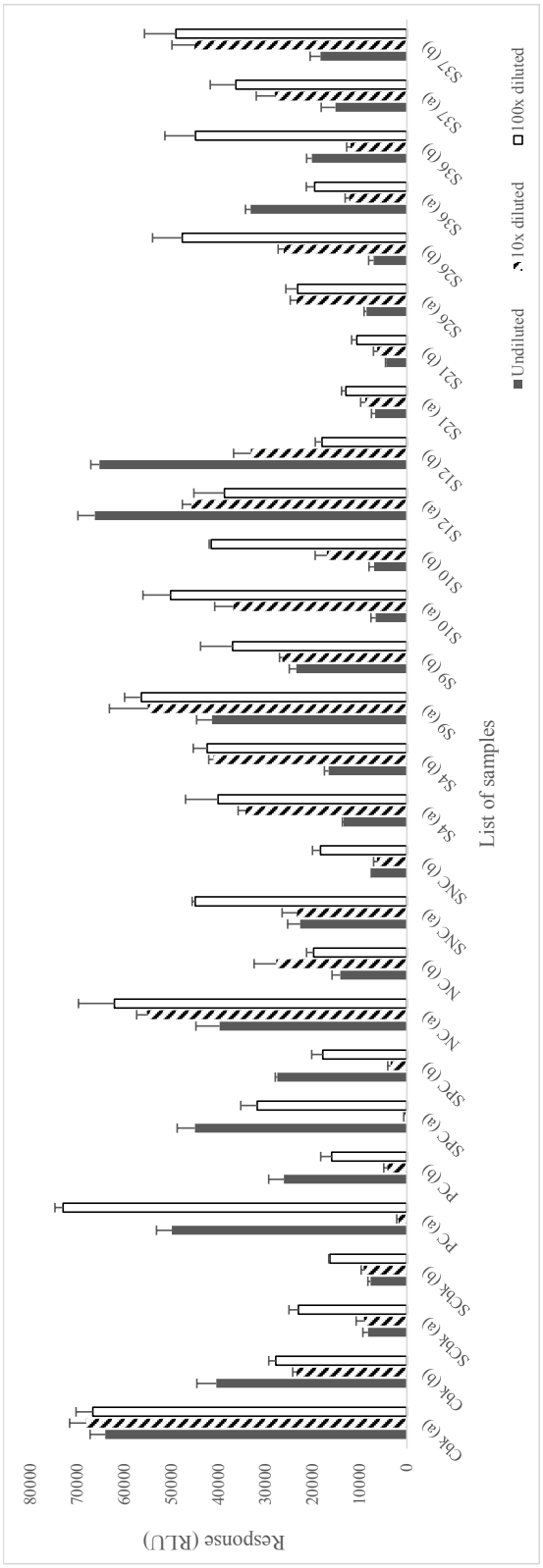

Figure S2a: Comparison of initial response (RLU) (a) to response of repeat analysis, 1 month after (b).

KEY: Cbk: Chemical blank; SCbk : Spiked chemical blank; PC : Positive control; SPC : Spiked positive control; NC : Negative control; SNC : Spiked negative control

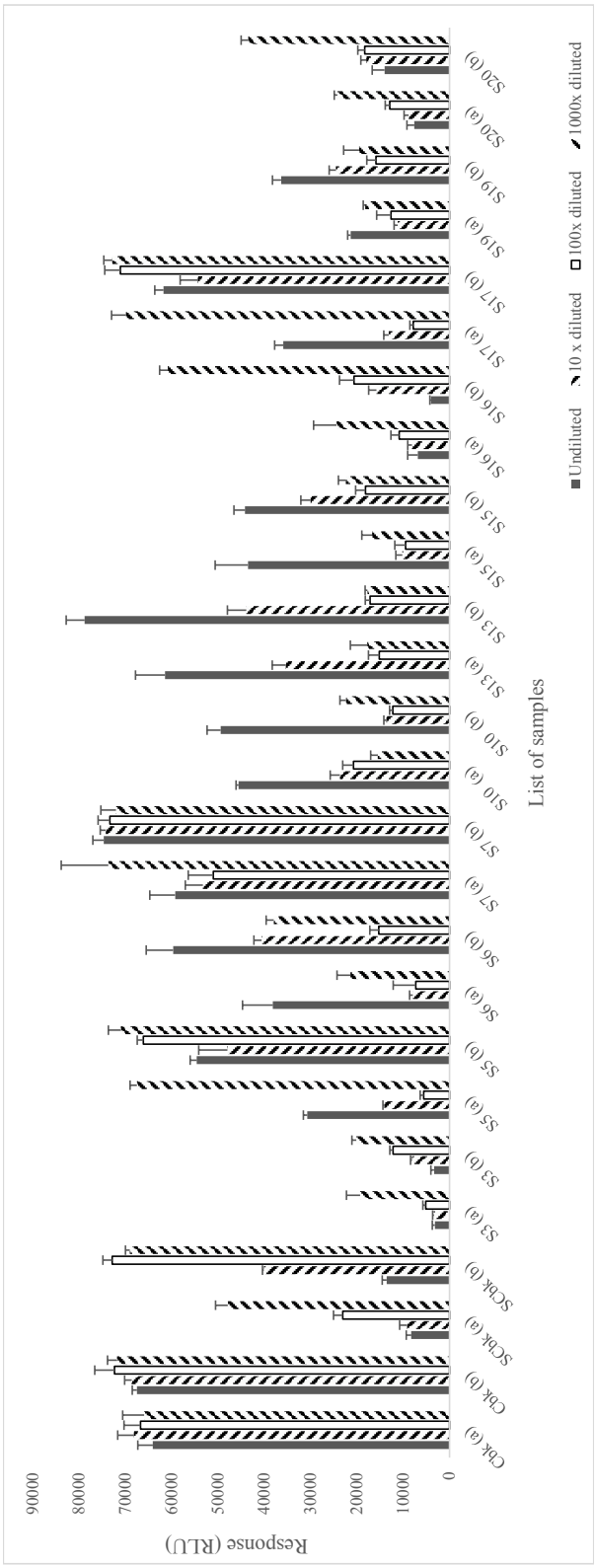

Figure S2b: Comparison of initial response (RLU) (a) to response of repeat analysis, 12 month after (b).

Cbk: Chemical blank; SCbk : Spiked chemical blank; PC : Positive control; SPC : Spiked positive control; NC : Negative control; SNC : Spiked negative control

3

Chapter 3

Identification of phosphodiesterase type-5 (PDE-5) inhibitors in herbal supplements using a tiered approach,

and associated consumer risk

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Abstract

The use of herbal supplements for improving sexual performance is a common practice amongst the youth and some senior citizens in Ghana. These products considered 'all natural', are highly preferred due to the perceived safety of natural products. However, the high adulteration rate of these products often compromises their safety.

Forty herbal supplements exhibiting various inhibition potentials in a previous PDE-Glo bioassay study were further investigated using different chemical analytical procedures to examine if the observed potencies were the result of inherent plant constituents or that of adulterants.

Results from the LC-MS showed the presence of sildenafil in almost all the supplements, with compound concentrations ranging from trace levels to above 100 mg sildenafil equivalents. Thirteen out of the 40 supplements contained adulterants with estimated daily intakes (EDIs) above 1 mg sildenafil equivalents and six out of the 13 even resulting in EDIs above 100 mg sildenafil equivalents. Only one sample contained a natural ingredient (icariin), nonetheless, the concentration (0.013 mg/g) of this compound was too low to explain the observed response by the bioassay. The estimated compound concentrations for thirty-five supplements based on the bioassay results were comparable to those of the LC-MS/MS results, however, discrepancies were observed for five supplements.

For one of the samples which showed discrepancies, a tiered approach, where the PDE-Glo bioassay was combined with LC-MS/MS and ^{1}H-NMR, revealed the presence of a sildenafil analogue not yet included in the LC-MS reference library.

3.1 Introduction

In an era where the ability of a man to sexually satisfy his partner(s) is considered a great competence, the opposite is regarded as a major impediment on his physical abilities and met with disdain. This negatively affects the individual's self-esteem and influences his general well-being, driving some even to the point of committing suicide [3]. Erectile dysfunction, regarded as the inability of a man to achieve and maintain an erection for a satisfactory sexual performance, is a global issue which has existed for decades but has gained much attention in recent years due to its prevalence (Aytac et al. 1999; Patel et al. 2014).

Various interventions ranging from physical therapy (counselling), lifestyle changes, surgeries and the use of drugs are recommended for the treatment and/or management of ED (Levine, 2000; Heidelbaugh, 2016). Most of these drugs approved for treatment of ED are known as phosphodiesterase type-5 inhibitors (PDE-5i). As the name suggests, these drugs inhibit PDE-5 enzyme activity in the corpus cavernosum, resulting in the accumulation of cGMP which subsequently promotes downstream relaxation of smooth muscle cells, allowing adequate blood flow to the penis, thereby enhancing erection. Examples of approved PDE-5i include, sildenafil citrate, tadalafil, vardenafil hydrochloride, and avanafil. There are also available analogues of these compounds which manufacturers often use for adulteration purposes (Low et al. 2009; Venhuis and De Kaste, 2012; Kee et al. 2018). The herbal based sexual enhancers are common in most African settings and are much preferred over the synthetic alternatives mainly due to their natural origin. Meanwhile, reports from different studies have indicated that most of these herbal aphrodisiacs are adulterated with either approved synthetic PDE-5i and/or their analogues (Reeuwijk et al. 2014; Kee et al. 2018). Table 1 shows the prevalence of adulteration of herbal aphrodisiacs in different studies.

[3] https://www.ncbi.nlm.nih.gov/pmc/articles/PMC3746995/ (accessed on 13/11/2020)

Approved PDE-5i are prescription-only drugs that are used strictly upon medical recommendation with appropriate monitoring. Their use as adulterants in herbal products raises legal issues as well as public health concerns. This is especially the case when unapproved drug analogues are used as adulterants as studies have shown that although these analogues may function similarly as the parent drug, differences in their chemical structures may alter their pharmacokinetic and pharmacodynamic profiles, including their toxicity (Venhuis and De Kaste, 2012; Buckle et al. 2017). Furthermore, PDE-5i are strongly contraindicated in patients using organic nitrate drugs, α-adrenergic blockers and CYP3A4 inhibitors (Kostis et al. 2005; Langtry and Markham, 1999). Their combined intake may synergistically promote increased relaxation of smooth muscle cells, resulting in a drastic reduction in systemic blood pressure (Kostis et al. 2005; Kloner, 2007). The sudden drop in blood pressure (hypotension) may possibly lead to other cardiovascular effects and in extreme cases, sudden shock or even death (Langtry and Markham, 1999; Gur et al. 2013). Patients advised against PDE-5 inhibition drugs may resort to herbal alternatives due to the belief that they can safely induce the same pharmacological effect while avoiding expected adverse effects (Blok-Tip et al. 2004). Nevertheless, due to their high prevalence of adulteration, consumers may unknowingly expose themselves to unanticipated risks (Poon & Lam 2007; Bakota et al. 2017).

Table 1. Prevalence of adulterated herbal products with active pharmacological ingredients (APIs).

Country	No. of samples analysed / No. of samples adulterated (%)	No. of sample adulterated		References
		With approved PDE-5i identified (%)	With analogues of PDE-5i identified (%)	
France	150 / 92 (61)	41 (45)	51 (55)	Gillard et al. 2015
India	85 / 1 (1)	1 (100)	-	Singh et al. 2010
Malaysia	62 / 34 (55)	Few [b]	Majority [b]	Bujang et al. 2017
Netherlands	71 / 23 (32)	1 (4)	22 (96)	Reeuwijk et al. 2014
Romania	50 / 11 (23)	8 (73)	3 (27)	Popescu et al. 2017
Singapore	175 / 134 (77)	123 (92)	11 (8)	Koh et al. 2009
USA	26 / 15 (58)	3 (20)	12 (80)	Mans et al. 2013
USA	353/166 (47)	166(100)	-	Tucker et al., 2018
Korea	404/130 (32)	-	-	Lee et al., 2021

[b] Insufficient information provided to derive actual number

Several LC-MS based techniques are employed for the identification and quantification of adulterants commonly used in natural products (Gratz et al. 2004; Petal et al. 2014). However, due to the on-going process of producing new analogues, these existing techniques may not be able to identify compounds unavailable in the reference library (Stranska-Zachariasova et al. 2019). As a result, these compounds may pass unnoticed in the LC-MS system, and products likely declared negative, i.e. free of adulterants, although they may be present. A way to overcome this challenge is by using a tiered approach where products will first be screened using a bioassay to assess their bioactive components, before further analysis and characterization by combined chemical analytical methods.

A previous study conducted on a series of 40 herbal supplements from the Ghanaian market revealed that 36 (90%) were able to inhibit PDE-5 enzyme activity according to a PDE-Glo bioassay (Akuamoa et al. 2021). Based on the estimated daily intakes (EDIs), 11 out of the 36 positive supplements were categorized as low (L; < 25 mg day-1), 16 as medium (M; 25 – 100 mg day-1) and 9 as high (H; > 100 mg day-1). A daily dose from 25 mg was regarded as the dose required for

a pharmacological effect, however, a dose above 100 mg could potentially lead to serious adverse health effects.

In the present study, all 40 herbal supplements were analyzed using various LC-MS based techniques to identify and quantify the compounds responsible for the observed inhibition potentials in the previous study. This was to conclude if the inhibition potentials were the result of inherent plant constituents or that of active pharmaceutical ingredients (API) intentionally added by producers to potentiate the desired outcome. This was also to show the accuracy of the bioassay in predicting the inhibition potentials of selected supplements, and to demonstrate as a proof-of-concept the advantages of employing a tiered approach involving the application of combined biological and chemical analysis in investigation of herbal products.

3.2 Material and methods

3.2.1 Supplements

The same samples tested in a previous study by Akuamoa et al. (2021) were further analyzed in this study. Table *S1* in the previous study presents the selected 40 supplements, their allocated sample ID, origin, and instructions for use. Based on their inhibition potentials determined by the PDE-Glo bioassay and corresponding estimated daily intakes (EDIs), supplements were categorized either as negative (N), low (L), medium (M) or high (H) intake (*Table 1;* Akuamoa et al. 2021).

3.2.2 Chemicals and reagents

Compounds used in this study were purchased from ChemCruz (UK and USA) and Carbosynth (UK) with purity between 95 and 99% unless indicated otherwise. Hydroxythiohomosildenafil (HTHS) (purity > 98%) was purchased from Toronto Research Chemicals (Toronto, Canada). Acetonitrile (Lot No. 1332591) and methanol (Lot. 1328761) (HPLC Supra-Gradient) were purchased from Biosolve, (Valkenswaard, The Netherlands), acetic acid (Lot. 1.00063) (100%) and formic acid (Lot. 1.00264) were from Merck (Darmstadt, Germany) and ammonium formate (Lot. 17843) was from Fluka, (Munich, Germany). Sterile syringe filters (w/0.45 µm cellulose acetate membrane) were purchased from VWR International (North America) and Whatman 0.2 µm pore size Mini-Prep^{TM} PTFE filter media with Polypropylene housing (CAT. US203NPUORG) and syringeless filter devices were from G.E Healthcare (Buckingham, UK). Ultra-pure water was prepared using a Milli-Q water purification system (Ref. A^{+}). The PDE-Glo phosphodiesterase assay kit (Promega, CAT No. V1361) was acquired from Fisher Scientific (Madison, WI, USA), Phosphodiesterase 5A1 human recombinant (CAT No. E9034) and 3-isobutyl-1-methylxanthine (IBMX) (CAT No. I5879) were purchased from Sigma-Aldrich (Saint Louis, USA) and Coaster 96-well, flat bottom, non-treated, non-sterile white polystyrene assay plates from Corning (NY, USA).

3.2.3 Sample pre-treatment

Refer to sample pre-treatment under materials and methods (2.2.3) in the previous study by Akuamoa et al. (2021) for detailed information. Prior to confirmation and quantification of compounds, extracts

were initially screened using the high resolution full scan orbitrap mass spectrometry (HR-FS-LC(Orbitrap)-MS) to identify a broad range of natural and synthetic compounds. For this, 250 µL of each sample extract was aliquoted into dilution vials and 250 µL extraction solvent (1% acetic acid in ACN:H_2O (80/20 (v/v)) was added. Mixtures were homogenized and filtered through a w/0 .45 µm cellulose acetate membrane syringe (VWR International (North America)). Next, 50 µL of each filtrate was pipetted into a 500 µL capacity integrated filter vial while 450 µL extraction solution was added and mixed. The resulting sample extract was analyzed in positive and negative mode with and without fragmentation using the HR-FS-LC-(Orbitrap) MS (data not shown). The HR-FS-LC-(Orbitrap) MS identified thirteen different compounds. Calibration curves of identified compounds were then prepared for quantitative analysis using the LC-MS/MS.

3.2.4 Sample preparation for identification and quantification of compounds

Two-hundred and fifty microliters (250 µL) of each extract was aliquoted into new dilution vials while 250 µL extraction solvent (1% acetic acid in MeOH:H_2O: ACN (70:20:10 (v/v)) was added. Solutions were homogenized and filtered through a w/0.45 µm cellulose acetate membrane syringe. Extracts were further diluted 10-, 100- and 1000-fold with MeOH: H_2O (50/50 v/v) for LC-MS analysis. Peak areas were quantified by means of external calibration curves (5, 10, 25, 50, 100, 250, 500 and 1000 ng/mL) using available commercial standards of the compounds identified during the screening process. The equation derived from the calibration curves were used to determine compound concentration in each supplement and expressed in mg sildenafil equivalents per gram or mL of the sample using the relative potency (REP) values established in a study by Bovee and others (unpublished) (Table *S2*). These REP values were derived from an *in-vitro* experiment by dividing the IC_{50} of sildenafil by the IC_{50} of the PDE-5i. The established IC_{50} was based on how much of the compound (PDE-5i) was needed to induce the same effect as sildenafil *in-vitro* by 50% (Hoetelmans R.M., 2011). For supplements with more than one PDE-5i identified, their individual concentrations was multiplied by the respective relative potency (REP) value of the respective PDE-5i and summed up to obtain the total concentrations. expressed in mg sildenafil equivalents per gram or mL of sample.

3.2.5 LC-MS/MS operating conditions for quantification and confirmatory analysis

Confirmation analysis was performed based on an in-house protocol with slight modifications (Biesterbos et al. 2019). The liquid chromatography-tandem mass spectrometry (LC-MS/MS) system consisted of an injection and pump system from Shimadzu (Hertogenbosch; The Netherlands) coupled to an Applied Biosystems (AB) Sciex QTRAP 5500 mass spectrometer (W.M., USA), operated in the ESI+ mode. The analytes were eluted through an Atlantis T3 (Waters, 3.0 x 100 mm) LC column, connected to a SecurityGuard C18 precolumn (Waters, 20 × 4.0 mm ID). The elution program consisted of two mobile phases: 5.0 mM ammonium formate prepared in ultrapure deionized water (A) and methanol (B), both containing 0.1% formic acid. The LC gradient started with 90% solvent A for 1 min, followed by a steep change to 100% solvent B for 8 min. It was maintained at 100% solvent B for 5.5 min and reverted to 90% solvent A for 6.5 min, making a total runtime of 20 min. The column was maintained at a constant temperature of 30 ºC and equilibrated with the initial mobile phase composition for at least 270 s before running the next injection. Elution flow rate was 0.40 mL min^{-1}. Detection was performed in MRM-mode with a probe temperature of 500°C, an entrance potential (EP) of 10 V, a decluttering potential of 96 V, and a dwell time of 10 ms. The retention times of icariin, sildenafil, tadalafil and vardenafil were 8.19, 7.59, 8.34, and 7.64 min. respectively. The precursor ions of icariin, sildenafil, tadalafil and vardenafil were selected as (*m/z*) 677.2, 475.1, 390.2 and 489.0, while the product ions were selected as (*m/z*) 369.0, 100.2, 135.1 and 169.0 with the collision energies (CE) of 43, 32, 22 and 40 (V) respectively. Data analysis was performed with Microsoft Excel version 2016.

3.2.6 UHPLC operating conditions for fractionation of chromatograms

Isolation of unknown PDE-5i in one of the supplements (S13) was carried out following the protocol of Lee and others (2009) with modifications. The same sample extract prepared for the confirmation analysis was used without dilutions. Fractionation of chromatograms was performed using a Kinetex 2.6 µm Polar-C18 100 A (50 X 2.1 mm) LC column (Phenomenex, NL) fitted to a Nexera X2 U(H)PLC from Shimadzu (Tokyo, Japan). Two mobile phases were used, i.e. 100% ultrapure deionized water (A) and 100% ACN (B). The runtime was set at 15 minutes and fractions were repeatedly collected at

a specified time frame based on peaks expressed as a function of time. The ACN phase of the collected fractions was evaporated under a continuous stream of nitrogen at 40 ºC. The residual solutions were initially stored at -20 ºC for 2 hr, then at -80 ºC for 30 min. Next, fractions were freeze dried overnight and then evaporated at -54 ºC under 0.047 mbar pressure in a vacuum. Extracts were then stored at -20 ºC for further analysis. A portion of the stored fractions was reconstituted in DMSO and re-tested in the PDE-Glo bioassay to estimate their inhibition potentials levels.

3.2.7 Sample analysis using the PDE-Glo bioassay

The PDE-Glo bioassay was performed following the protocol described in a previous study by Akuamoa et al. 2021. Briefly, a 5 µL aliquot of sample extract, 7.5 µL PDE-5 enzyme and 12 µL cGMP (20 µM) were each pipetted into a 96-well plate, was mixed and incubated in the dark for 90 min. A termination solution (12 µL) containing termination buffer + 100 mM 3-isobutyl-1-methylxanthine was used to terminate the reaction after the incubation period. Next, detection solution (12 µL) consisting of detection buffer + protein kinase was added, mixed for 5 min and incubated for another 20 min. Finally, 50 µL kinase glo reagent containing kinase glo substrate + kinase glo buffer was added and incubated for another 10 min. The Biotek Synergy HT (Vermont, USA) was used to measure luminescence signals in relative light units (RLUs). Data analysis and graphs were plotted using Microsoft Excel (2016). Active fractions that showed high inhibition potential were subsequently analyzed using the time-of-flight mass spectrometry (TOF-MS).

3.2.8 LC-TOF-MS operating conditions

Active fractions identified by the PDE-Glo bioassay were divided into two portions of 45 µL each and analyzed using the HPLC system (Agilent 1200 Series) coupled to a Bruker micro-TOF mass spectrometer, operated in ESI positive and negative mode. For analysis, 5 µL of dissolved active fractions in ACN:H_2O (80:20 v/v %) was injected through an Altima HP C18 column (155 x 2.2 mm). Two mobile phases consisting of 100% ultrapure deionized water (A) and 100% ACN (B) were used to set the elution gradient at a flow rate of 1 mL min^{-1}. The mixture of solvents is noted as A/B (v/v). The HPLC running elution gradient started with 80% solvent A 80/20 to 60/40 for 10 min and changed

to 40/60 in 15 minutes. After that, the solvents went back to starting conditions (80/20) in 20 minutes and equilibrated for another 15 minutes . It was then decreased to 60, 40 and 20% each for 5 min, making a total run time of 260 mins with an elution flow rate of 1 mL min^{-1}. During the measurement, the detection was performed in the mass range of 99 to 1501 m/z with a mass resolution of 15000 FWHM. Peaks with the highest intensity were collected and their masses were recorded. Next, collected fractions were dried, and their constituents' structure elucidated using ^{1}H-NMR.

3.2.9 Compound identification using ^{1}H-NMR

The structure of the identified mass was elucidated by ^{1}H-NMR analysis following an in-house protocol. The dried fractions purified by the Bruker TOF LC-MS were evaluated using a Bruker Avance III 600 MHz NMR spectrometer, 3 mm NMR tubes and a cryogenic NMR probe. Quantitative ^{1}H-NMR spectra were recorded after dissolving the dried fraction in 200 µL DMSO-D6 and 2400 scans read using an 1D NOESYGPPR pulse sequence. In addition to the 1D 1H NMR data, 2D NMR datasets, 2D 1H-1H COSY and 2D 1H-1H TOCSY were assessed as well.

3.2.9.1 Verification and confirmation of newly identified constituents

The identified compound was verified by spiking the active fraction with the pure compound. Analysis of pure compound, the unspiked and spiked active fraction was carried out concurrently using the TOF-MS. Peaks from the unspiked active fraction was compared to the peaks of the spiked and pure compound for a possible match. Similar peaks were mixed and re-analyzed for confirmation. The identity of the unknown compound was confirmed based on co-elution of peaks of the mixed sample showing similar mass spectrum, increased intensity and a well-defined molecular mass (Figure *S*1).

3.3 Results and discussion

Forty herbal supplements collected from various markets and pharmacies in Accra (Ghana) were previously analyzed using the PDE-Glo phosphodiesterase bioassay to estimate their PDE-5 inhibition potentials (Akuamoa et al. 2021). According to the results obtained, it was evident that 36 (90%) of the selected supplements had the ability to inhibit PDE-5 enzyme activity at varying degrees. Based on the estimated daily intakes (EDIs) of supplements, eleven out of the 36 positive supplements were categorized as low intakes (L; < 25 mg), 16 as medium intakes (M; 25 – 100 mg) and 9 as high intakes (H; > 100 mg).

In the current study, all 40 supplements were subjected to further investigations using combined chemical analytical procedures, first of all to establish if the observed inhibition potentials were caused by inherent plant constituents or the effect of synthetic PDE-5i used as adulterants, and secondly to confirm the accuracy of the bioassay in guiding the selection of supplements of major concern. The calibration curves of compounds were used to determine their concentration in each supplement and expressed in mg sildenafil equivalents per gram or mL of sample using the so-called To this end the concentration of each compound in the sample was multiplied by its REP value to sildenafil (REP=1) and summed up as the total concentration in mg sildenafil equivalents per gram or mL of supplement. A cut-off of 1 mg/g was selected for sildenafil as reporting level, as lower concentrations could be the result of cross-contamination from supplements with very high levels during analysis. Besides, only higher levels clearly pointed at purposeful adulteration to obtain the desired outcome, therefore, low levels were considered less relevant. For other analogues, a cut-off of 0.1 mg/g was applied since cross-contamination was less likely and with regards to the higher REPs for some of them. It should be considered that using these cut-offs may result in the elimination of supplements where a relatively low level might still result in an intake larger than 25 mg per day due to a high daily dose (especially in the case the liquids samples).

Table 2 shows only the results for supplements with concentrations above the cut-off limits and their corresponding EDIs. The concentration of sildenafil in 11 supplements (28%), were above 1 mg/g. Considering other PDE-5i, three supplements contained low amounts of tadalafil, vardenafil and traces

of other compounds but below 0.1 mg/g. A natural PDE-5i known as horny goat weed (icariin as the active ingredient), with multiple functions (such as increasing nitric oxide synthesis in the penis, inhibiting PDE-5 in smooth muscle, mimicking endogenous androgens, correcting endothelial dysfunction, including treatment of multiple cardiovascular diseases (He et al., 1995; Xin et al., 2003; Xu and Huang, 2007; Shindel et al., 2010; Fang, J., & Zhang, Y. (2017)), was detected in one supplement (S13) although the concentration was below the cut-off limit. This implied that none of the supplements contained natural PDE-5i. Considering the recommended daily doses of supplements, 11 out of the 40 would resulted in intakes (EDI) at pharmacologically relevant levels (i.e. > 25 mg/day). For S20 this is mainly the result of the relatively high recommended daily dose. Due to high sildenafil concentrations, the EDIs for six supplements were above 100 mg, which were likely to cause adverse effects. Although traces of other compounds were detected, sildenafil was the most identified adulterant. This may partly be attributed to the readily available manufacturing process of sildenafil making it easy to reproduce (Terrett et al. 1996; Buckle et al. 2017; Kee et al. 2018).

Table 2. Identified compound(s) in supplements, their total compound concentrations, recommended daily doses, and estimated daily intakes (EDI).

	Compound concentration (mg per g or mL)			Total compound concentration (mg per g or mL)	Average dose (g/day)	No. of doses per day	EDI (mg sildenafil equivalents per day)
	Sildenafil	Tadalafil	Vardenafil				
Sample ID	1*	0.1*	2*				
S3	13			13	1.01	2	26
S6	123	2		123	0.47	2	116
S10	243	0.2		243	0.46	2	223
S14	14			14	1.16	4	64
S15	322			322	0.36	6	696
S16	18			18	0.65	2	23
S19	135			135	0.43	2	116
S20			1	2	10	3	60
S21	67			67	0.97	4	268
S23	2			2	20	1	40
S37	89	2		89	2.03	4	723
S38	16			16	1.47	4	94

* relative potencies (REP) of compounds

The estimated concentrations in each supplement as determined by the LC-MS/MS were subsequently compared to the estimated concentrations based on their inhibition potentials in the previous PDE-Glo bioassay. Table *S2* shows the complete list and Table 3 only shows the comparison for supplements categorized as "medium" or "high" intake based. It should be noted that sample dilution in the previous study influences the estimated levels and that only a few of the samples were tested at a 10,000-fold dilution (not used for estimating daily intakes). However, for comparison with the LC-MS/MS result, the 10,000-fold dilution was included when available. The concentrations of all supplements (S1, S2, S8, S9, S11, S12, S18, S26, S29, S31, S32, S33, S34, S36, S40) classified as "negative" or "low" intake in the PDE-Glo assay were below the established cut-off limits for all compounds. This proved that there was no false-negative results indicated by the bioassay .

Table 3. Comparison between estimated concentrations in the PDE-Glo bioassay and the LC-MS/MS analysis. Only supplements classified as medium or high intake according to the bioassay are shown.

Sample ID	Bioassay concentration (mg/g)			LC-MS/MS Concentration (mg/g)	Evaluation
	100*	1000*	10000*		
S3	> 8	36	532	13	Unidentified compounds
S4	1			< 1	Comparable
S5	> 8	< 1		< 1	Comparable
S6	> 8	31	299	123	Comparable
S10	3	47	377	243	Comparable
S13	5	40	873	< 1	Unidentified compounds
S14	4	13		14	Comparable
S15	> 8	43	444	322	Comparable
S16	7	26		18	Comparable
S17	> 8	< 1		< 1	Comparable
S19	6	39	326	135	Comparable
S20	6	26		2	Unidentified compounds
S21	5	29		69	Comparable
S22	2			< 1	Comparable
S23	1			2	Comparable
S24	2			< 1	Comparable
S25	3			< 1	Comparable
S27	> 8	< 1	< 7	< 1	Comparable
S28	4	< 1		< 1	Comparable
S30	2			< 1	Comparable
S35	4	6		< 1	Unidentified compounds
S37	6	32	278	89	Comparable
S38	6	50	127	16	Unidentified compounds
S39	4	< 1		< 1	Comparable

* dilution factor applied in the PDE-Glo Bioassay
Empty means that no clear inhibition was observed in the PDE-Glo Bioassay

Comparison of results between the two methods showed that for 19 out of the 24 supplements, the responses from the bioassay could be explained by the LC-MS/MS results. This included 3 extracts (S5, S17, S27) which according to the bioassay indicated levels above 8 mg/g (based on a full inhibition at the 100-fold dilution) and below 1 mg/g (based no inhibition at the 1000-fold dilution). For seven other supplements, the estimated levels based on the 100-fold dilution was quite low and LC-MS/MS

analysis did not detect the presence of any PDE-5i. However, for the remaining 9 supplements the high bioassay responses could well be explained by the level of sildenafil, considering the variations in the estimated levels of the different dilutions. Nevertheless, the comparison also revealed discrepancies between the estimated amounts by the bioassay and the LC-MS/MS for five supplements (S3, S13, S20, S35 and S38), for which the results indicated the presence of unknown active PDE-5i constituents. Example, the concentration estimated by the bioassay in S13 based on the 1000- and 10,000-fold dilutions, was 40 and 873 mg/g respectively, whereas the amounts of known PDE5i determined by LC-MS/MS were below the cut-off limit. The disparity. pointed at the presence of unknown active compounds in these supplements but not yet detected in the more targeted LC-MS/MS based methods (possibly due to the absence of these compounds in the reference library).

One of the supplements which showed discrepancies in results between the two analysis (S13) was selected for further investigations. This was to demonstrate as a proof-of-concept, the advantages to using a tiered approach where a biological assay precedes a more targeted analytical approach, thus exploring the benefits of both methods. The purpose therefore was not to analyze all five supplements with discrepancies, but rather to demonstrate the accuracy of the bioassay in providing a positive lead for a follow-up analysis using various chromatographic techniques. This was aimed at the identification of other PDE-5i in S13, that were not (yet) identified by the LC-MS/MS based technique. To this end an activity-based fractionation was performed using UHPLC. Fractions containing peaks observed in the chromatogram at 190 nm were collected, as schematically presented in Fig. 1 and subsequently analyzed in the PDE-Glo bioassay. Overall, ten different fractions (peaks a-j) were repeatedly collected and pooled. Peak ‘c’ was the highest and most obvious peak.

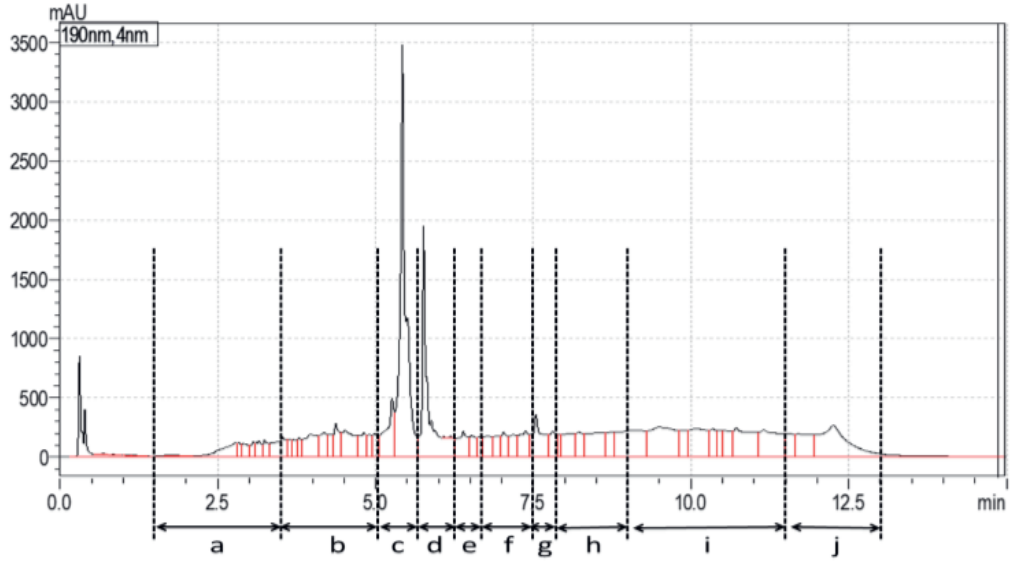

Figure 1. UHPLC chromatogram of S13 extract and its fractionation pattern.

The collected fractions were subsequently analyzed in the PDE-Glo bioassay to access their PDE-5 inhibition potentials. The principle of the assay results in a lower response (measured in relative light units (RLU) when inhibition of PDE-5 activity by the active component in the fraction is high, and vice versa (Akuamoa et al. 2021). Regarding the absence of peaks, fractions h, i and j (Fig. 1) were pooled together and tested as a single fraction. Figure 2 shows the response of the tested fractions in various dilutions. The results obtained indicated that fraction c displayed the strongest inhibition potential and was therefore selected for further analysis of its constituent(s).

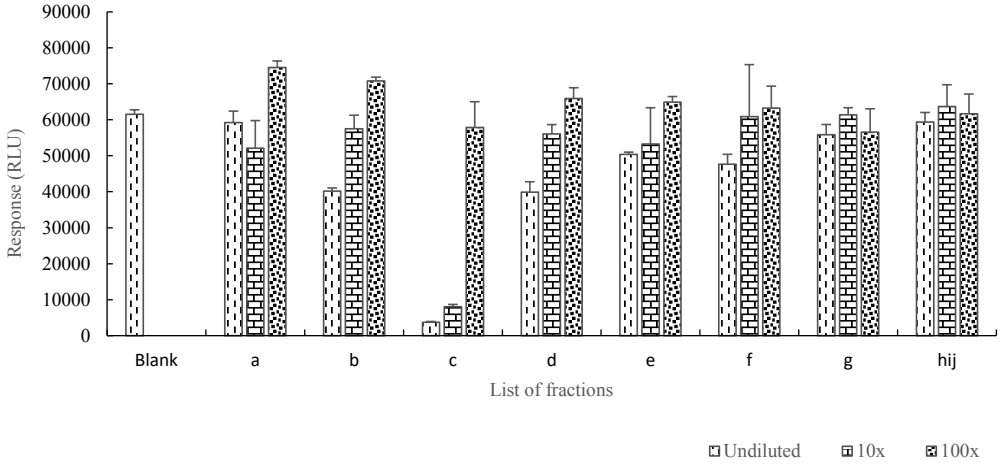

Figure 2. Inhibition potentials of collected fractions of S13 tested in the PDE-Glo bioassay.

Using the time-of-flight (TOF)-MS for the analysis of fraction c, the presence of a compound with mass in the positive and negative mode (i.e. $[M+1]^+$ and $[M - 1]^-$ at *m/z* of 521.2 and 519.2 respectively), was elucidated. This pointed at a neutral mass of 520.7 *m/z* and a corresponding molecular formula $C_{23}H_{32}N_6O_4S_2$. To further evaluate the unknown constituent, fraction c was analyzed by ^{1}H-NMR. Comparing the NMR data obtained to the NMR spectrum of sildenafil, it was found that the compound was an analogue of sildenafil having a hydroxyethyl group instead of a methyl group attached to the piperazinyl nitrogen and the oxygen atom replaced with a sulfur atom in the pyrazolopyrimidine moiety. This compound was identified as hydroxythiohomosildenafil, which is in line with the mass and structural composition derived from the TOF-MS data.

To confirm this, the commercially available compound was purchased and analysed by TOF-MS. The results from the pure compound showed similar retention time and mass spectrum as the newly identified constituent. Next, a dose response curve for the activity of hydroxythiohomosildenafil in the PDE-Glo bioassay was constructed to establish its relative potency to sildenafil (Fig. 3). The results obtained revealed hydroxythiohomosildenafil to be substantially more potent than sildenafil with an IC_{50} of 80 nM as compared to the IC_{50} of 900 nM for sildenafil. This implied that hydroxythiohomosildenafil was about 12-fold more potent than its parent compound (i.e. sildenafil).

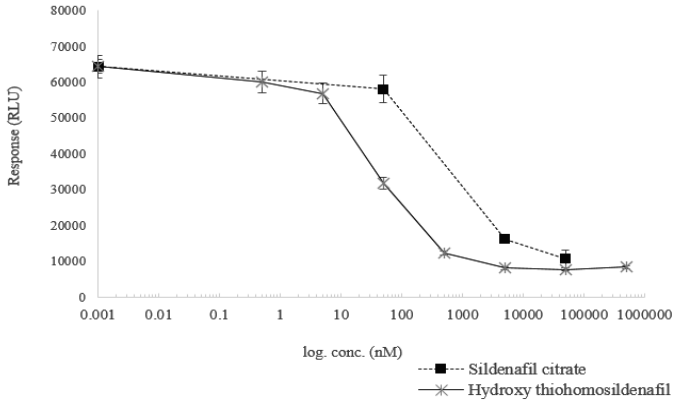

Figure 3. Concentration dependent reduction in PDE-5 activity. IC_{50} values of 900 nM and 80 nM were calculated from the fitted dose-response curves for sildenafil and hydroxythiohomosildenafil, resulting in a REP value of about 12 for hydroxythiohomosildenafil.

The reference library of the HR-FS-LC-(Orbitrap)MS was then updated to include hydroxythiohomosildenafil and the MS method optimized to also quantitatively detect this additional PDE-5i. Next, the concentration of hydroxythiohomosildenafil in S13 was estimated in sildenafil equivalents considering its relative potency factor (RPF). The estimated concentration according to the LC-MS/MS was 12 mg sildenafil equivalents, which was in line with that of 10 mg sildenafil equivalents based on the effect of the 1000-fold dilution in the bioassay. This implied that, upon inclusion of hydroxythiohomosildenafil in the reference library, the chemical analysis did not miss this compound. The use of this analogue with a substantially higher intrinsic PDE5i activity than sildenafil may raise concerns, especially when used in higher dose levels, since already at a dose of >8 mg it would give rise to exceedance of the highest therapeutic dose of 100 mg sildenafil equivalents.

Although sildenafil is effectively metabolized in the liver by CYP3A4 (major route) and CYP2C9 (minor route), the combined intake of PDE-5i and CYP3A4 inhibitors may affect this process resulting in blood concentration levels of PDE-5i (Huang and Lie 2013; Graziano et al. 2017). Instances of sildenafil-related hepatic toxicity have been reported in a study by Graziano et al. (2017), where a 65-year-old, a 49-year-old and a 58-year-old suffered similar fate in 2003, 2005 and 2009 respectively. Although high concentrations of PDE-5i in supplements may not result in fatal conditions on their own, pre-existing

conditions such as hypertension and diabetes may likely potentiate the effects of these compounds and may even result in death (Matheeusen et al. 2015; Kee et al. 2018). Such was the fate of a 66-year-old man who was found dead in a hotel room and was discovered in his possession empty pockets of various types of PDE-5i which were suspected to be his cause of death.[4] Although in principle supplements with intakes above 25 mg are expected to elicit a pharmacological response, those above 100 mg may potentially cause some adverse effects. However, it is equally important to address samples with EDIs below 25 mg sildenafil equivalents. This is because individuals with certain medical conditions such as pulmonary hypertension are treated with Revatio, which has sildenafil as the active ingredient and a starting dose of 10 mg/ mL (Oral suspension) and 20 mg for the tablet.[5]

3.4 Conclusions

This study addressed the drawbacks regarding the use of chemical analysis only (i.e chromatographic techniques) for identification and quantification of adulterants in herbal products. The LC-MS/MS only identified and quantified compounds that were already in the reference library, aiding the possibility of missing non-targeted compounds. The advantages of using a tiered approach, where a biological assay is first used to screen supplements in order to assess their bioactive components, followed by LC/MS confirmation and quantification, is best suited for botanical and botanical preparations. The focus of this study therefore was not to investigate all five supplements with discrepancies to identify the compounds causing the inhibition, but rather to demonstrate as a proof-of-concept, the use of a tiered approach, where the results of the bioassay served as a positive lead towards the selection of relevant supplements for further characterization using chemical analysis.

Overall, 13 out of 25 (54%) supplements which showed medium (M) and high (H) inhibition potentials in the previous study (Akuamoa et al. 2021), were adulterated with synthetic PDE-5i, with estimated

[4] https://www.ghanaweb.com/GhanaHomePage/NewsArchive/66-year-old-man-dies-in-a-hotel-room-as-his-25-year-old-side-chick-vanishes-1138130 (accessed on 20/3/2021)
[5] https://www.revatio.com/taking-revatio (accessed on 12/5/2021)

daily intakes of six supplements being above 100 mg sildenafil equivalents, thus exceeding the highest safe dose of Viagra (sildenafil).

Collaboration

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Declaration of interest statement

Authors declare that they have no competing financial interests or personal relationships that could influence the work reported in this paper.

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Supplementary Tables

***Table S1**:* Relative potency (REP) values of PDE-5i established in a study by Bovee and others (unpublished)

Compound	Relative Potency value
Sildenafil	1
Vardenafil	2
Tadalafil	0,1
Hydroxyvardenafil	45
Hydroxythiohomosildenafil	42
Thiosildenafil	41
Thiodimethylsildenafil	37
Hydroxy acetildenafil	18
Propoxyphenylsildenafil	15
Mirodenafil	13
Thiohomosildenafil	11
Acetildenafil	2
Dimethylsildenafil	1
Hydroxyhomosildenafil	0,7
Dimethyl acetildenafil	0,4
Avanafil	0,3
Aminotadalafil	0,1
Icariin	0,002
Acetaminotadalafil	0,001
Yohimbine	n/a

Table S2: Comparison between concentrations in supplements as determined by the PDE-Glo assay and LC-MS/MS analysis, expressed in mg sildenafil equivalents per gram (or mL) of sample and their corresponding estimated daily intake.

	Bioassay Concentration (mg/g)				
Sample ID	100*	1000*	10000*	LC-MS Concentration (mg/g)**	Inference
S1	< 0.1			<1	Comparable
S2	4	< 1		<1	Comparable
S3	> 8	36	532	13	Unidentified compounds
S4	1			<1	Comparable
S5	> 8	< 1		<1	Comparable
S6	> 8	31	299	123	Comparable
S7	< 0.1	< 1		<1	Comparable
S8	< 0.1			<1	Comparable
S9	< 0.1			<1	Comparable
S10	3	47	377	243	Comparable
S11	1	5		<1	Comparable
S12	2	< 1		<1	Comparable
S13	5	40	873	<1	Unidentified compounds
S14	4	13		14	Comparable
S15	> 8	43	444	322	Comparable
S16	7	26		18	Comparable
S17	> 8	< 1		<1	Comparable
S18	< 0.1			1	Comparable
S19	6	39	326	135	Comparable
S20	6	26		2	Unidentified compounds
S21	5	29		69	Comparable
S22	2			2	Comparable
S23	1			<1	Comparable
S24	2			<1	Comparable
S25	3			<1	Comparable
S26	4	10		<1	Comparable
S27	> 8	< 1	< 7	<1	Comparable
S28	4	< 1		1	Comparable
S29	3	< 1		<1	Comparable
S30	2			<1	Comparable
S31	4	< 1		<1	Comparable
S32	3	< 1		<1	Comparable
S33	3	< 1		<1	Comparable
S34	2	< 1		<1	Comparable
S35	4	6		N/D	-
S36	1	1		<1	Comparable
S37	6	32	278	89	Comparable
S38	6	50	127	16	Unidentified compounds
S39	4	< 1		<1	Comparable
S40	3	< 1		<1	Comparable

*dilution factor **It is assumed that total concentrations below the cut-off are below 1 mg/g

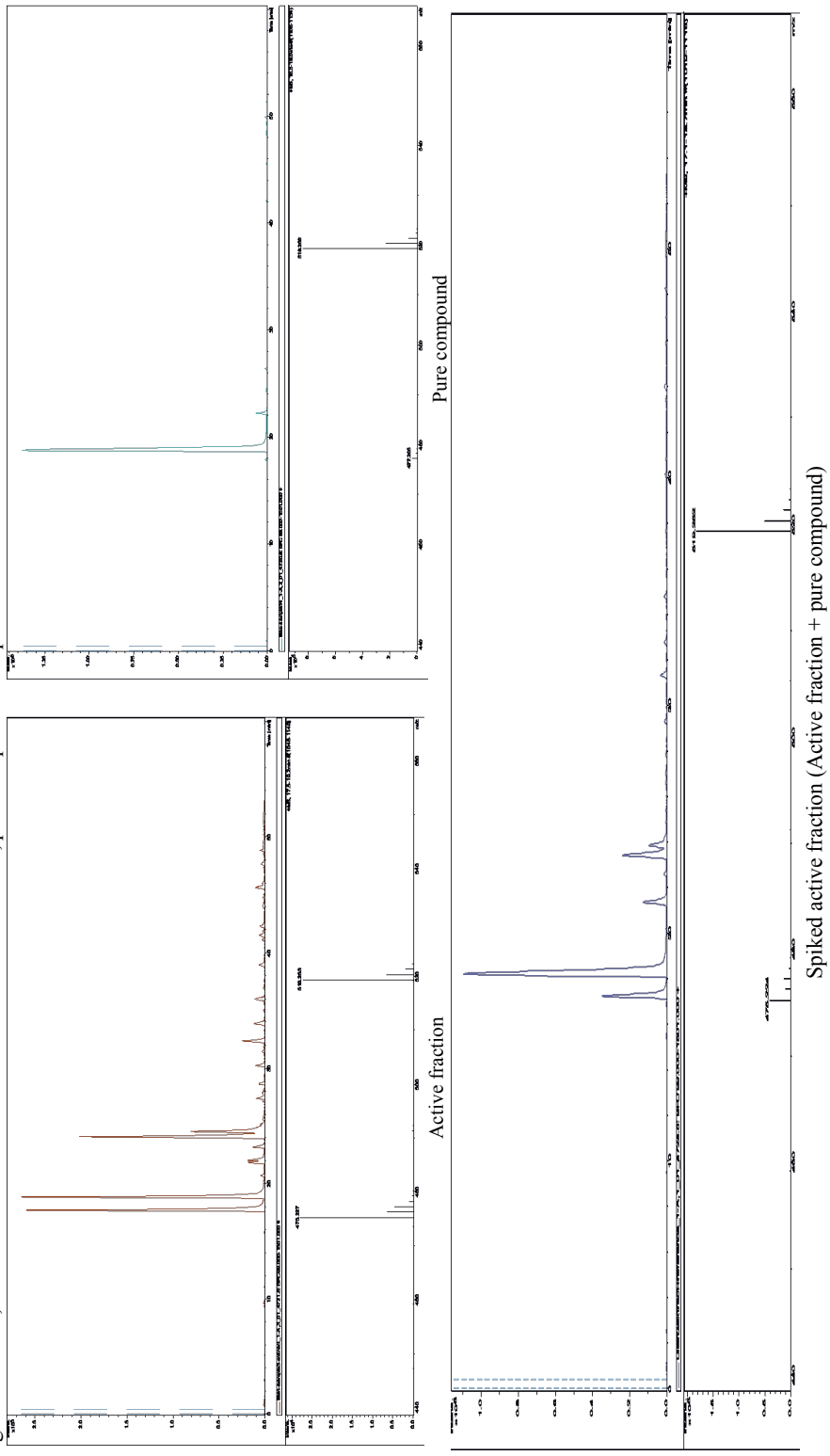
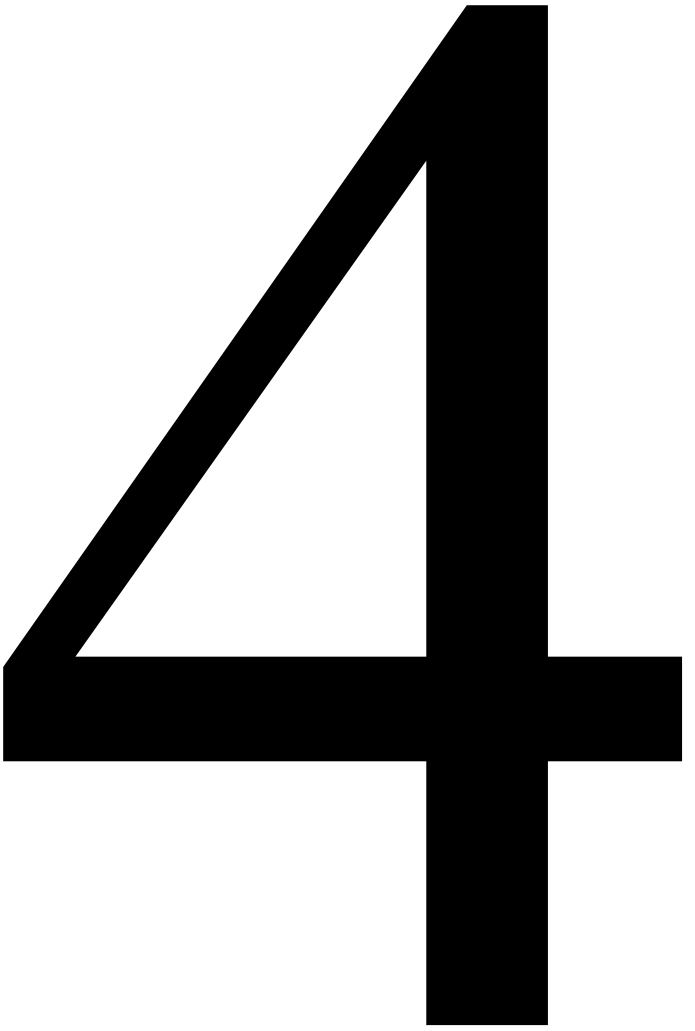


Figure S1:Peaks, *m/z* ratios and retention time of the active fractions, pure compound and spiked active-fractions



Chapter 4

Presence and risks of polycyclic aromatic hydrocarbons, dioxins and dioxin-like PCBs in plant supplements as elucidated by a combined DR CALUX® bioassay and GC-HRMS based approach

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Abstract

Plant based supplements may contain undesirable contaminants such as polycyclic aromatic hydrocarbons, dioxins and dioxin-like polychlorinated biphenyls (dl-PCBs) due to the sources of raw materials use or processing methods applied. The presence of these contaminants in a series of herbal supplements, sold on the Ghanaian market for improved sexual performance, was examined using the DR CALUX® bioassay in combination with GC-HRMS analysis.

Cell responses at 4 and 48 hrs exposure to extracts prepared without an acid-silica clean-up were higher than those obtained from extracts with an acid-silica clean-up. This indicated that the 40 supplements contained only low levels of stable aryl hydrocarbon receptor (AhR) agonists like polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs) and dl-PCBs, while some contained less stable AhR-agonists.

Ten out of the 40 supplements selected for confirmation with GC-HRMS analysis were found to contain PCDD/Fs and dl-PCBs at levels varying from 0.01 to 0.19 pg toxic equivalent (TEQ)/g only, whiles the sum of 4 polycyclic aromatic hydrocarbons (Σ4PAHs), representing less stable AhR agonists, ranged from not detected (ND) to 25.5 ng/g. These concentrations were in line with the responses observed in the DR CALUX® bioassay. The concentration of PCDD/Fs and dl-PCBs corresponded to estimated daily intakes (EDIs) ranging from 0.01 to 1.20 pg TEQ/day, or 0.001 to 0.12 pg TEQ/kg bw/week for a 70 kg bw consumer. These exposure levels were below the established tolerable weekly intake (TWI) of 2 pg TEQ/kg bw/week, thus indicating low concern for consumers' health. Similarly, the EDIs based on the detected Σ4PAHs in supplements ranged from 7.2 to 111 ng/day, or 0.1 to 1.6 ng/kg bw/day, corresponding to MOE values above 10 000, indicating a low health concern.

Keywords: DR CALUX®, GC-HRMS, PAHs, dioxins and dl-PCBs, plant supplements, AhR-agonist, Ghana.

4.1 Introduction

In most parts of the world and especially in Africa, individuals depend on herbal medicinal plants and supplements as an alternative to their primary healthcare needs. Also, in Ghana, there is substantial use of plant-based supplements due to socio-cultural and economic reasons. These products come in different forms (e.g. decoctions, powders, liquids, capsules, tablets, ointments, and creams) and are available at various retail shops (e.g. open markets, kiosks, pharmacies and chemical shops) across the country. To extend their shelf-life, the majority are sun dried or smoked over open fires in order to reduce the moisture content before being sold in their crude state or further processed into other forms. These production processes may pre-dispose finished products to certain undesirable contaminants such as polycyclic aromatic hydrocarbons (PAHs), dioxins (polychlorinated dibenzo-p-dioxins and dibenzofurans (PCDD/Fs)) and dioxin-like polychlorinated biphenyls (dl-PCBs) when conditions (such as high temperature, low oxygen) and applied fuels are favorable for their formations (Lohmann et al., 2000; Hoogenboom et al., 2012). Also, these contaminants may occur in herbal products when harvesting raw materials from polluted/contaminated sites including production amidst heavily industrialized areas (Schuler et al., 1997; Loufty et al., 2010).

Human exposure to a mixture of PAHs, dioxins, and dl-PCBs have gained much attention from the World Health Organization (WHO, 2002) and other relevant authorities (IPCS, 2003; Zelinkova, Z., & Wenzl, T., 2015). The abundant nature of these compounds in the environment make their elimination from the food chain difficult. However, all efforts and resources are being directed towards their mitigation, through regulation of their formation and adequate processing of chemicals, products and foods, in order to minimize their occurrence in the environment and food chain.

The PCDD/Fs of concern are those composed of two benzene rings with chlorine atoms (four to eight, at least at positions 2, 3, 7 and 8), linked together by one or two oxygen atom(s). The toxicity and level of persistence of these compounds are dependent on the number and position of the chlorine atoms. The name dioxin-like PCBs relates to compounds with structural similarities to PCDD/Fs and similar mode of action, proceeding via stimulation of the aryl hydrocarbon receptor (AhR) pathway

(Nebert et al., 1993; White and Birnbaum, 2009; Tuomisto, 2019). The intensity at which these agonists bind to the AhR, inducing the related effects determine their relative toxic potencies, where 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) is considered the most toxic congener. The WHO (2006) therefore selected 2,3,7,8-TCDD as an index chemical and assigned it a Toxic Equivalency Factor (TEF) of 1. The TEF for six other PCDDs, ten PCDFs and twelve dl-PCBs were derived from *in vitro* and *in vivo* studies relative to the index chemical (i.e. 2,3,7,8-TCDD) (Van den Berg et al., 2006). In practice, the determined concentrations of the individual congeners are multiplied by the TEF values for each congener and summed to derive the toxic equivalents (TEQ) content.

PAHs consist of only carbon and hydrogen with two or more aromatic rings arranged in a linear, cluster or angular form (Abdel-Shafy and Mansour, 2016). Although PAHs are easily metabolized by cytochrome P450 enzymes, they are of concern, as some of their metabolites are more toxic than the parent compound itself. For instance, the metabolic activation of PAHs in mammalian cells leads to the formation of dihydrodiol epoxides which can bind covalently to DNA, thereby initiating DNA replication errors and mutations (Cerniglia, 1993; Billiard et al., 2002). Other concerns include interferences with the thyroid gland, reproductive and immune systems (Veraldi et al., 2006; Oostingh et al., 2008; Ahn et al., 2016).

The EU Scientific Committee on Food have identified 15 PAHs of concern due to their carcinogenicity and mutagenicity (SCF, 2002). Initially, benzo[*a*]pyrene (BaP) was used as the golden standard for assessing the risks of PAHs and product limits. Later on, the EFSA (2008) concluded that the sum of 4 PAHs (Σ4PAHs) (i.e. benzo[*a*]anthracene, benzo[*a*]pyrene, benzo[*b*]fluoranthene and chrysene) is more representative of the levels of carcinogenic PAHs in food, which was then implemented in legislation. Besides their mutagenic effect, certain PAHs also can stimulate the AhR (EFSA 2008). As a result, the Dioxin Responsive Chemical-Activated LUciferase gene eXpression (DR CALUX®) bioassay, initially developed for the detection of PCDD/Fs and dl-PCBs, is also suitable to screen for the presence of PAHs (Bovee et al., 1996). Moreover, this bioassay can distinguish PAHs from PCDD/Fs and dl-PCBs due to differences in their

metabolic stability after prolonged exposure, as well as the use of a selective clean-up (i.e. an acid-silica column) for PCDD/Fs and PCBs (Bovee et al., 1998).

Currently in Ghana, there is lack of data on consumer exposure to PAHs, dioxins and dl-PCBs through the consumption of plant-based supplements, although they are suspected to be contaminated with these compounds due to processing methods used (i.e. drying over open fires, smoking). Amongst the most popular supplements are those sold for improving sexual performance. Previous studies by Akuamoa et al. (2021) on these herbal supplements revealed their phosphodiesterase type 5 (PDE-5) inhibition potentials due to the presence of known and unknown synthetic PDE-5 inhibitors. Another study focused on the presence of pyrrolizidine alkaloids (Akuamoa et al., unpublished), a class of natural toxins present in certain plant families. The present study is intended to further investigate these herbal supplements with the aim of identifying other undesirable contaminants such as PAHs, dioxins and dl-PCBs, possibly introduced before or during production. To this end, the 40 supplements were screened using the DR CALUX® bioassay to identify the presence of stable (acid-silica clean-up and 48 hrs exposure) and less stable (without acid-silica clean-up and 4 hrs exposure) AhR-agonists. Based on the results from the bioassay screening, ten supplements were selected for further analysis using gas chromatography coupled with high resolution mass spectrometry (GC-HRMS) in order to evaluate whether the observed bioassay responses were due to known PAHs, dioxins and/or dl-PCBs, and if the levels of these compounds could explain the observed bioassay responses. Finally, the risk for consumers was assessed using the MOE approach for PAHs, and the established Tolerable Weekly Intake (TWI) for dioxins and dl-PCBs.

4.2 Materials and methods

4.2.1 Supplements

Forty herbal supplements sold for improved sexual performance were selected from various markets and pharmacy shops in Accra. These supplements were previously tested in a study by Akuamoa et al. (2021) to determine their inhibition potentials against PDE-5 enzyme activity, and the presence of synthetic PDE-5 inhibitors (Akuamoa et al., unpublished). In this study, the supplements were analyzed for the potential presence of genotoxic and carcinogenic AhR agonists (i.e. PAHs) including AhR agonists with other potential adverse effects (i.e. PCDD/Fs). Table *S1* in a previous publication presents the list of 40 supplements and their allocated sample ID, origin, instructions for use, and recommended daily intakes (Akuamoa et al. (2021).

4.2.2 Chemicals and reagents

Methanol (MeOH) ultra LC-MS (purity 99.98 %; CAS-67-56-1), n-hexane PEC grade (CAS-110-54-3), and diethyl ether (purity 99.5 %; CAS-60-29-7) were purchased from Actu-All Chemicals (Oss, The Netherlands). Na_2SO_4 (CAS-7757-82-6) was purchased from Merck (Darmstadt, Germany), silica from Ossum Chemicals (Oss, The Netherlands), and sulphuric acid from Merck (Darmstadt, Germany). The modified rat hepatoma cell line (H4IIE-luc) was obtained from Wageningen University (identical to the cell line from Bio Detection Systems BV, Amsterdam, The Netherlands). Alpha minimum essential medium (AMEM) (1x) and foetal bovine serum (FBS) qualified H1 (Lot. 08Q7183K) were purchased from Gibco (Grand Island, New York), penicillin (5000 U; Lot. 097M4877V) and streptomycin (5 mg/L) from Sigma-Aldrich Chemie (Schnelldorf, Germany), 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; CAS-1746-01-6) from Schmidt BV (Amsterdam, The Netherlands), dimethyl sulfoxide (DMSO) from Merck (Darmstadt, Germany), and benzo[a]pyrene (BaP) (purity 99 %; CAS-50-32-8) from Dr Ehrenstorfer (Augsburg, Germany). Cell culture lysis reagent was obtained from Promega Benelux BV (Leiden, The Netherlands). T75 cell culture flasks, and tissue culture treated white 96-well clear bottom assay plates with lid, were purchased from VWR

International BV (Amsterdam, The Netherlands). Ultra-pure water was prepared using a Milli-Q water purification system.

4.2.3 Acid-silica preparation

Silica (334 g) was impregnated with sulphuric acid (167 g) in a 1000 mL conical flask and activated by mixing overnight (14 hrs) on a head-over-head mixer (Heidolph Reax 2) at room temperature.

4.2.4 Sample preparation

One gram or millilitre portion of solid or liquid sample was weighed into a 15 mL capacity polypropylene eppendorf tube. As sample controls, feed samples containing a mixture of dioxins and dl-PCBs at concentrations of 0.02, 0.29, 0.48, 0.70, 1.57 and 3.35 ng TEQ/kg were included and treated in a similar manner as the supplements. Next, 4 mL methanol (MeOH) : H_20 (85:15 v/v %) was added and vortexed at speed 5 for a minute while ensuring that all solutes were in suspension and not stuck at the bottom (Vortex-2 Genie, Model G-56OE). Subsequently, 4 mL n-hexane/diethyl ether (97:3 v/v %) was added, and placed on a head-over-head mixer (Heidolph Reax 2) for 30 minutes at speed 5, and centrifuged (2910 g) for 10 minutes at 22ºC (Sorvall RC3BP plus low-speed centrifuge; Thermo Scientific). After centrifugation, the hexane/diethyl ether phase was collected into a clean borosilicate glass tube. Again, 4 mL of n-hexane/diethyl ether (97:3 v/v %) was added and the extraction process repeated. The hexane/diethyl ether phase was again collected and added to the previous one making a total volume of about 8 mL These extracts were treated with or without a clean-up over an acid-silica column before further analysis in the bioassay.

4.2.5 Sample treatment without an acid-silica clean-up

Each extract was evaporated to 1 mL using a vacuum evaporation system (Savant SPD 2010, Speed Vac Concentrator) with temperature set at 45ºC, ramp at 3, vacuum pressure at level 30 and a runtime of 15 minutes. Afterwards, 20 µL DMSO was added (as a keeper) to the 1 mL extract and mixed before further evaporation in the vacuum evaporation system using the same program but reduced runtime (until the n-hexane/diethyl ether (97:3 v/v %) solvent was evaporated). Finally, an additional

20 µL DMSO was added to the extracts and mixed, resulting in a total volume of around 40 µL DMSO.

4.2.6 Sample clean-up using acid-silica

Glass columns (held in place with a clamp on a ring stand) were prepared with tips directed towards the opening of 50 mL liquid scintillation vials. Next, moulds of cotton (treated to complete dryness at 160ºC) were pushed down to the tips of each glass column. Ten grams of acid silica was weighed into each glass column followed by 2 grams of Na_2SO_4 (treated at 125ºC). The side of each glass column was gently tapped to ensure both anhydrous mixtures were compacted. Next, each column was pre-washed with 20 mL hexane/diethyl ether (97:3 v/v %) to condition the column while getting rid of impurities. The 50 mL liquid scintillation vials were replaced by new ones and the entire content of each sample extract (approximately 4 mL) was pipetted (and rinsed twice with 2 mL hexane/diethyl ether (97:3 v/v %)) into the glass columns. Next, extracts were eluted with 20 mL hexane/diethyl ether (97:3 v/v %) and subsequently an extra 10 mL of the same eluent, making a total volume of about 40 mL.

Each 40 mL sample extract was evaporated to about 1 mL using a vacuum evaporation system (Savant SPD 2010, Speed Vac Concentrator) with temperature set at 45ºC, ramp at 3, vacuum pressure at level 30 and a runtime of 60 minutes. Afterwards, the 1 mL was withdrawn from the vial, rinsed twice with 2 mL hexane/diethyl ether (97:3 v/v %) and transferred to a 6 mL borosilicate tube which already contained 20 µL DMSO (as a keeper). The solution was mixed before evaporation in the vacuum system with the same program but reduced runtime. The n-hexane/diethyl ether (97:3 v/v %) solvent was evaporated, and an additional 20 µL DMSO were added to the extracts suspended in DMSO and mixed, resulting in a total sample volume of around 40 µL.

4.2.7 Controls on the evaporation process of extracts

In order to check to what extent the evaporation step affected the responses, both BaP and TCDD standards were treated in two different ways, i.e. one was evaporated and the other one not (see below). The “evaporated” TCDD and BaP standard solutions in DMSO were included and treated in

the same way as sample extracts right before the second evaporation, i.e. by adding 20 µL of each TCDD and BaP standard solution and 5 mL n-hexane/diethyl ether (97:3 v/v %) into a 6 mL borosilicate tube, mix and evaporate together with the sample extracts. The n-hexane/diethyl ether (97:3 v/v %) was evaporated, and an additional 20 µL DMSO was added to the standards suspended in DMSO and mixed, resulting in a total volume of about 40 µL.

4.2.8 DR CALUX® bioassay

Each extract and standard in DMSO was diluted with 2 mL culture medium, i.e. AMEM supplemented with 10 % FBS and 0.5 % penicillin/streptomycin, resulting in a final DMSO concentration of ≈ 2 %. The DR-CALUX bioassay was basically performed as described previously by Hoogenboom et al. (1999). In short, recombinant rat hepatoma cells (H4IIE-luc) were grown at 37ºC (5 % CO_2) and 100 % relative humidity in culture medium. Hundred microlitre cells (about 40 000 cells/well) were seeded in 96-well plates (Corning) and grown for 24 hrs before adding 100 µL of each extract or standard in culture medium in triplicate (final volume 200 µL, final concentration DMSO ≈ 1 %). To check the effect of the evaporation, in each series, a BaP and TCDD calibration curve was included, where 20 µl of the standard solution and 20 µl DMSO were added directly to 2 ml incubation medium, of which 100 µl were added to each well, in triplicate. The luciferase concentration was subsequently measured 4 and 48 hrs after exposure. For this, the medium was removed, and the cell monolayers were washed with 200 µL phosphate-buffered saline (PBS) (Oxoid). Cells were lysed using 20 µL cell culture lysis reagent (Promega) and incubated for 25 minutes at room temperature.

Luciferase activity of the cell lysate was measured with a CLARIOstar microplate reader (BMG Labtech) from Isogen Life Science BV (Utrecht, The Netherlands) which automatically added 100 µL assay mixture (substrate) containing 20 mM tricine, 1.07 mM $(MgCO_3)_4\,Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4.7H_2O$, 0.1 mM EDTA, 33.3 mM DTT, 261 µM Coenzyme A, 470 µM luciferin, and 530 µM ATP at a pH of 7.8. TCDD and BaP standard dose-response curves were fitted using a user-defined exponential equation y=a0/(1+(x/a1)^a2) (where a0 was the maximum response, a1 was the

concentration showing a half-maximal response (EC_{50}) and a2 was a coefficient for the steepness of the curve) with SlideWrite Plus v.6.1 (Advanced graphics software, USA). Graphs were plotted with GraphPad Prism 5.

4.2.9 GC-HRMS analysis of PAHs, dioxins and dl-PCBs

Depending on the available amount, 0.7 to 4 g (average 2.3 g) supplement were mixed with celite, spiked with ^{13}C labelled standards of PCDD/Fs, PCBs and PAHs, and extracted using accelerated solvent extraction (ASE). After filtering over Na_2SO_4, half of the extract was used for analysis of PAHs, and the other half for PCDD/Fs and PCBs.

The latter compounds were analyzed using standard and accredited methods (Ten Dam *et al.*, 2016). Extracts were concentrated, mixed with hexane and purified on a Dextech (LCTech), using an acid silica, alumina oxide and a carbon column, resulting in two fractions (i.e. fraction A with the eight mono-ortho- and six non-dioxin-like (ndl)-PCBs and fraction B with the 17 PCDD/Fs and four non-ortho PCBs). Both fractions were concentrated to 500 µL isooctane or toluene, respectively, and analyzed with GC/HRMS (Autospec), using a DB-5ms column (60 m x 0.25 mm ID: Fd 0.25 µm) (Agilent J&W, Folson, USA). The MS was tuned to a resolution of 10 000 at an electron energy of 35 eV and emission current of 600 µA. The required sensitivity to enable detection of 200 fg TCDD was a signal-to-noise ratio of 50. Of fraction A, 2 µL was injected in SSL mode at 280°C and of fraction B 100 µL on a PTV injector (Gerstel, Mülheim an der Ruhr, Germany) in Solvent Vent mode via a liner with glass pearls. The U.S. EPA protocol was used regarding the TEQ calculations with the data obtained from the GC-HRMS analysis. Absolute levels of each congener were multiplied by their individual WHO_{2005}-TEF which were then added together to obtain the overall PCDD/Fs and dl-PCB concentration expressed in TEQ (Van den Berg et al., 2006).

PAHs were analyzed using a routine and accredited method by concentrating the other half of the ASE-extract. After dissolving in cyclohexane/ethyl acetate 50:50, it was purified on a GPC column (Biobeads SX3). The relevant fraction was concentrated and purified using an alumina oxide column. The eluate was concentrated to 100 µL and analyzed by GC-HRMS using a Varian Select-PAH

column (30 m x 0.25 mm ID: Fd 0.15 µm). A volume of 1 µL was injected in SSL mode at 300°C and the MS was tuned to a resolution of 12,000 at an electron energy of 35 eV and emission current of 600 µA.

4.3. Results and discussion

The present study applies two procedures for the detection of stable and unstable AhR-agonists. One is based on the metabolic capacity of the cells used in the bioassay, the other is based on the application of an acid-silica clean-up which is known to remove especially less stable compounds from the extracts. Regarding the differences in the metabolic capacity of the engineered H4IIE rat liver cells used in the DR CALUX® bioassay for conversion of PCDD/Fs versus PAHs, responses were measured after 4 and 48 hrs exposure. This was to predict the potential metabolism and/or stability of AhR-active compounds present in the supplement extracts. Less stable AhR-agonist (e.g. PAHs) are known to be metabolized (inactivated) by cells, resulting in a relatively high response at 4 hrs and much lower or almost no response at 48 hrs, whereas more stable compounds (e.g. PCDD/Fs and dl-PCBs) will elicit a higher response after prolonged exposure (i.e. 48 hrs) due to their persistent nature (Bovee et al., 1996; Hoogenboom et al., 1999; Zhou et al., 2021). Fig. 1 shows the dose-response curves of BaP (Fig. 1a) and TCDD (Fig. 1b) at 4 and 48 hrs exposure. BaP elicited a clear dose-response when using an exposure time of 4 hrs. However, after prolonged exposure (i.e. 48 hrs) the response was lost at the lower concentrations and only observed at high concentrations. The responses obtained at 48 hrs with very high concentrations of BaP are most likely due to metabolic limitation of the cells, i.e. the capacity needed to fully metabolize (and inactivate) these large amounts of BaP.

In contrast, the shape of the dose-response curve of TCDD at 4 and 48 hrs was similar although signals at 4 hrs were lower than at 48 hrs (Fig. 1b). Compared to BaP, the dose-response curve of TCDD did not shift to the right with longer exposure time, which can be attributed to the stable and persistent nature of TCDD. The evaporation step during the sample pre-treatment did not affect the bioassay outcomes, as responses of ‘evaporated’ and ‘unevaporated’ BaP and TCCD standards were identical (data not shown). This implied that the evaporation procedure was optimal and loss of DMSO was minimal, which is of importance given that it was previously demonstrated that percentage of DMSO affects the luciferase production by the cells (Hoogenboom et al., 2006).

In addition, feed samples prepared with soy oil spiked with PCDD/Fs and dl-PCBs at varying concentrations (used in daily routine when screening samples for enforcement purposes) were included in order to check for unexpected matrix effects when testing extracts with an acid-silica clean-up (Figure 1c). The results showed a dose-related response at 4 and 48 hrs exposure. Both responses at 4 and 48 hrs showed similar characteristics as the TCDD standard curve, i.e. no clear shift to the right. The results also demonstrated the successful extraction and clean-up of PCDD/Fs and dl-PCBs over acid-silica and the sensitivity of the assay to determine levels around the EU maximum threshold for the sum of PCDD/Fs and dl-PCBs in feed (i.e. 1.25 ng TEQ/kg) (Commission Directive 2006/13/EC).

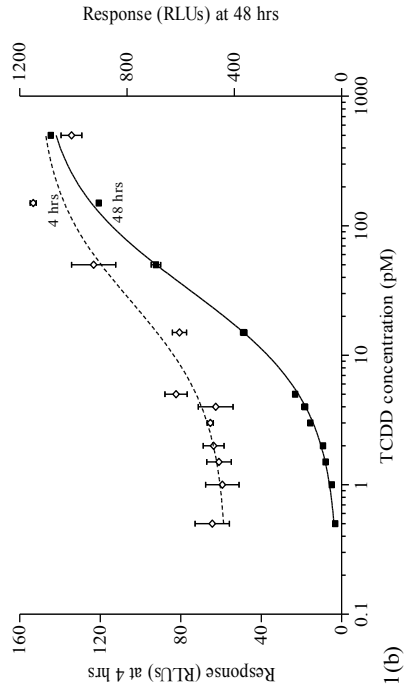

Fig. 1(b)

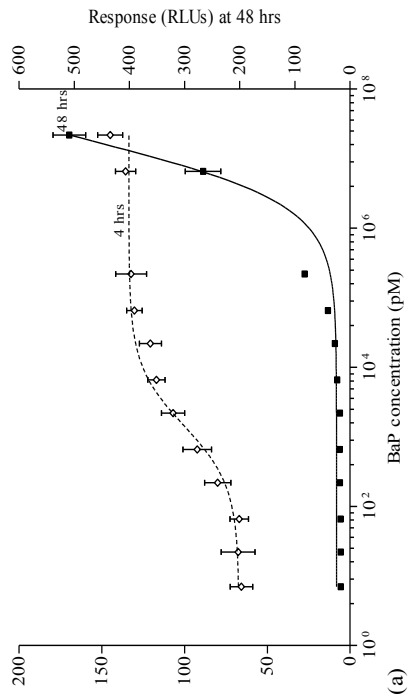

Fig. 1(a)

Fig. 1(c)

Fig.1. DR CALUX® cell responses at 4 and 48 hrs exposure to BaP (1a), TCDD (1b), and extracts of feed samples (cleaned over acid-silica columns; with each different data point representing a different extract with a different concentration of a mixture of PCDD/Fs and dl-PCBs for which the concentration in TEQ was determined after GC-HRMS analysis (1c)). Results show mean (± SD; n=3) responses in relative light units (RLUs).

Figure 2 shows the cell responses at 4 and 48 hrs upon exposure to extracts prepared with and without an acid-silica clean-up. Exposure for 4 hrs to extracts prepared without an acid-silica clean-up was to identify less stable compounds that do not survive this clean-up, whereas cells exposed for 48 hrs to extracts treated with acid-silica was to identify the presence of more stable/persistent compounds. The average response of each extract at 4 and 48 hrs was corrected for the average response observed for DMSO at 4 hrs and 48 hrs. These corrected average responses are shown in Fig. 2.

Cell responses at 4 hrs for 35 (87.5 %) sample extracts prepared without an acid-silica were higher than the responses obtained from extracts prepared with an acid-silica clean-up, with an overall average response of 120 RLUs compared to 39 RLUs, respectively (Fig. 2a). This meant that the responses were mainly caused by compounds that were not recovered from the acid-silica column. However, cell responses at 4 hrs for five sample extracts (S7, S18, S20, S25, S35) out of the 40 supplements treated with and without acid-silica after 4 hrs exposure were similar, indicating the presence of compounds that were able to survive the acid-silica clean-up.

At 48 hrs, extracts treated with an acid-silica column generally did not show elevated responses (average response of 12 RLUs), thus indicating the absence of stable AhR-agonists like dioxins and dl-PCBs. This implied that the AhR-agonists detected after 4 hrs in the five supplement extracts (S7, S18, S20, S25, S35) purified over an acid-silica column, were completely metabolized or inactivated by the rat liver cells at 48 hrs (Fig. 1b). Interestingly, cell responses of some extracts i.e. S1, S10, S14, S36 and S40 not treated with an acid-silica clean-up were even higher than at 4 hrs (Fig. 2b). These samples likely contained compounds that were not, or only partly metabolized by the cells (as observed for BaP in high concentrations at 48 hrs), but were lost upon acid-silica clean-up.

Generally, the results from the DR CALUX® assay indicated that none of the 40 supplements contained high levels of stable AhR-agonists, but rather high levels of less stable AhR-agonists which were poorly metabolized by the cells. Based on the results obtained, ten supplements (i.e.

S1, S10, S14, S34, S36, and S40 which showed high responses at 4 and 48 hrs exposure without acid silica), two supplements, S7 and S25 (which resulted in equal responses at 4 hrs with or without acid-silica), and two other supplements (S22 and S29) (with low responses at 48 hrs without acid silica) were selected for further characterization using GC-HRMS to enable evaluation of the outcomes of the bioassay. The idea for this selection was not to test all samples but rather show as a proof, the accuracy of the bioassay in presenting reliable leads as a screening assay.

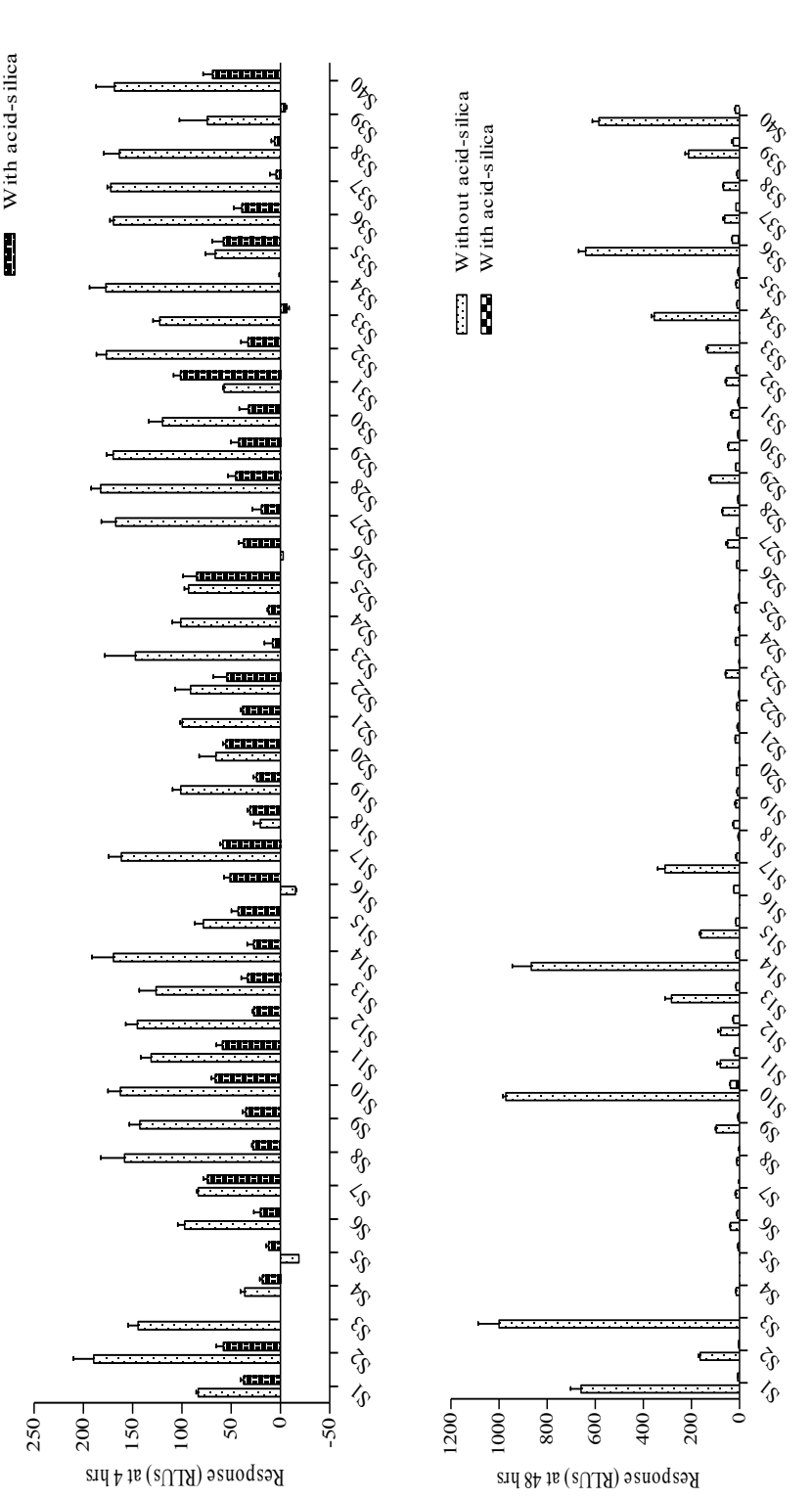

Figure 2. Responses at 4 (a) and 48 hrs (b) exposure to sample extracts prepared with and without acid-silica-clean up (mean ± SD; n=3 incubations for single clean-up).

The GC-HRMS reference library contained 24 PAHs including the 4 EU marker PAHs (i.e. benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene and benzo[*a*]pyrene) proposed by the WHO (2005) and EFSA (2008) as indicative for PAHs with genotoxic and carcinogenic properties. Table *S1* shows the list of 24 PAHs and their respective concentrations in nanogram PAH per gram of supplement. Amongst the list of PAHs, phenanthrene was abundantly identified in all ten samples with concentrations ranging from 2.98 to 94.30 ng/g. Fluoranthene and pyrene also contributed significantly to the sum of 24 PAHs.

In order to assess the risk of consumers upon exposure, individual concentrations of the 4 EU marker PAHs were summed to obtain the sum of 4 PAHs (Σ4PAHs), which ranged from not detected (ND) to 25.5 ng/g (Table 1). Amongst the 4 EU marker PAHs, benzo[*b*]fluoranthene occurred the most (8 times) and recorded the highest single PAH level in the ten supplements, followed by benzo[*a*]pyrene (7 times) in terms of occurrence and chrysene in terms of concentration. The six supplements (S1, S10, S14, S34, S36, and S40) which showed high cell responses at both 4 and 48 hrs (without acid silica), showed detectable levels of PAHs according to the GC-HRMS analysis. In addition, S7, S22 and S25 which showed low responses at 4 and 48 hrs (without acid-silica), showed low levels of the PAHs determined by the GC-MS analysis. S29 which induced a high response (170 RLUs) at 4 hrs but relatively low response (120 RLUs) at 48 hrs (both without acid-silica), recorded the highest Σ4PAHs (25.5 ng/g).

The GC-HRMS measured Σ4PAH concentrations (ND to 25.5 ng/g) was converted to their respective RLUs using the respective BaP dose-response curves at 4 and 48 hrs (data not shown). The highest concentration of 25.5 ng/g (S29) resulted in a medium concentration of about 25 nM, which was expected to elicit a response of 153 RLUs at 4 hrs and 35 RLUs at 48 hrs. The predicted responses (RLUs) for the six other supplements with detectable but lower PAH levels (S1, S10, S14, S34, S36, and S40) also explained a substantial part of the DR CALUX® response at 4 hrs but not at 48 hrs. This indicates that the high responses observed after 48 hrs for some of the supplements (without acid-silica), may be the cause of other (natural) AhR-agonists, which were poorly metabolized by the

cells but were lost upon use of an acid silica clean-up (Amakura et al., 2002; Jeuken et al., 2003). An example of such compounds are the furocoumarins that occur in e.g. citrus fruits (van Ede et al., 2008).

Table 1. Compound concentrations (ng/g) for the Σ4PAHs as determined by GC-HRMS analysis.

PAHs	S1	S7	S10	S14	S22	S25	S29	S34	S36	S40
Benzo[a]anthracene	1.80	ND	2	ND	ND	ND	7.28	ND	ND	10.10
Chrysene	2.83	0.11	2.46	5.97	ND	ND	11.50	ND	ND	ND
Benzo[b]fluoranthene	1.11	0.14	2.03	3.65	ND	ND	3.97	2.84	3.50	6.81
Benzo[a]pyrene	0.66	<0.10	1.35	2.83	ND	ND	2.78	1.11	1.46	5.98
Σ4PAHs	6.4	0.25	7.84	12.45	ND	ND	25.53	3.95	4.96	22.89

ND represent not detected

To estimate the daily intakes (EDIs) that would result from use of the supplements, the Σ4PAH levels were multiplied by the average weight of the recommended dose and by the number of doses per day (Table 2). The EDIs for supplements with detected levels ranged from 7.2 to 111.3 ng Σ4PAH /day. Dividing the EDI by the average weight of a consumer (assuming an average weight of 70 kilogram) the estimated intake levels ranged from 0.10 to 1.59 ng Σ4PAH /kg bw/day (Table 2).

Because of the genotoxic and carcinogenic nature of PAHs, the margin of exposure (MOE) approach was used to assess the potential health risks upon exposure to these contaminants upon daily long-term use of the respective herbal supplements. The $BMDL_{10}$, representing the lower 95% confidence interval of the dose that causes a 10% increase in tumor incidence, was used as a reference point. The MOE is the ratio between this $BMDL_{10}$ and the estimated human daily intake (EDI). For PAHs, the European Food Safety Authority (EFSA, 2008) established a $BMDL_{10}$ of 340 µg/kg bw/day as a reference point for the 4 EU marker PAHs based on a carcinogenicity study on coal tar mixtures by Culp et al. (1998). Thus, the MOE was determined by dividing the $BMDL_{10}$ (340 000 ng/kg bw/day) by the EDI (ng/kg bw/day) (Table 2). For compounds with a genotoxic and carcinogenic mode of

action, the EFSA considers an MOE larger than 10 000 as a low health concern whereas those close to or less than 10 000 indicate a potential concern for consumer health and a possible priority for risk management actions (EFSA 2005, 2008). The calculated MOE values for the supplements based on the EDIs were all above 10 000 (Table 2), thus indicating a low concern for consumer health.

Table 2. Calculated daily intakes of the Σ4PAHs resulting from daily use of the supplements and MOE values.

Sample ID	Σ4PAHs (ng/g)	Avg. weight dose (g)	No. of dose/day	EDI (ng/day)	EDI (ng/kg bw/day)	MOE
S1	6.40	1.07	4	27.4	0.39	868 867
S7	0.25	30	4	29.9	0.43	796 519
S10	7.84	0.46	2	7.2	0.10	3 299 689
S14	12.45	1.16	4	57.8	0.83	411 993
S22	<0.4*	30	3	<36	<0.50	>680 000
S25	<0.4*	60	1	<24	<0.34	>1 000 000
S29	25.53	1.09	4	111.3	1.59	213 816
S34	3.95	1.34	4	21.2	0.30	1 124 126
S36	4.96	0.96	2	9.5	0.14	2 499 160
S40	22.89	1.14	2	52.2	0.75	456 033

* below the limit of quantification.

Although the levels of PAHs identified in this study was relatively low compared to other studies, the reported levels indicate the prevalence of PAHs in herbal products. For instance, Krajian and Odeh (2013) investigated the presence of the 16 US (EPA) PAHs in 10 medicinal plants in Syria and reported a concentration range of 47 to 890 µg/kg using reverse-phase high-performance-liquid-chromatography (HPLC) coupled to both ultraviolet and fluorescence detection. Also, Cui et al., (2014) investigated seventy-nine herbal medicinal products for the presence of 16 PAHs using a similar chemical analytical approach as employed in this study, and reported PAH concentrations ranging from 21.1 to 2856 µg/kg which were higher than the amounts determined in the present study. Similarly, Zhang et al., (2012) investigated nine Chinese medicinal herbs (Panax, Fructus liquidambaris, liquorice root, mulberry twig, cassia seed, eucommia bark, rose flower, indigowoad leaf and fleeceflower root) for the presence of 16 genotoxic and carcinogenic PAHs and recorded concentrations ranging from 98.2 - 2245 µg/kg. Fiedler et al. (2002) even recorded amounts from

497-1162 mg/kg when analyzing the total PAHs content in four Chinese herbal teas (two green teas, and two brick teas).

It has been suggested that estimating exposure based on the relative potency factors (RPF) of PAHs is more representative of individual compound contributions towards the final estimations than estimations based on the absolute PAH concentrations (Nisbet and LaGoy 1992). A potency-based approach for risk assessment on PAHs would help in reducing the tendency of overestimating the risk, since several PAHs seem relatively less potent (i.e. low RPF) when compared to BaP. Nevertheless, the challenge in applying these RPFs is the lack of universally accepted RPFs applicable when estimating combined exposure levels for risk assessment, which is as a result of the different assays employed in the various studies and the resulting variations in the RPFs established. For instance, Machala and others (2001) established the RPFs of PAHs based on a DR CALUX assay, Villeneuve et al. (2002) employed the *in vitro* ethoxyresorufin-*O*-deethylase assay, while Pieterse et al. (2013) employed the PAH CALUX assay, thus contributing to differences in the established RPFs. These may subsequently result in great variations in estimated intakes when expressed in e.g. benzo(a)pyrene-equivalents (BaP) and the final conclusions drawn. It is also worth noting that reported RPFs for PAHs are based on skin tumours (following skin exposure) or *in vitro* assays, and there are not sufficient data to establish RPFs for oral exposure. As a result, the use of RPFs in estimating PAH intakes to assess health risks of consumers has not been adopted by relevant authorities and organizations such as the WHO, EFSA and the United States Environmental Protection Agency (USEPA). Instead, EFSA preferred to base the assessment on four relatively potent PAHs present in PAH mixtures and assumed equal potencies.

Regarding the PCDD/Fs and dl-PCBs, the concentration of individual congeners and the sum of 7 PCDDs, 10 PCDFs, and 4 non-ortho PCBs in the selected supplements as measured by the GC-HRMS analysis are presented in Table *S2*. Mono-ortho and non-dioxin-like PCBs were not detected in any of the samples. In most of the supplements, the concentrations of individual congeners were below the LOQ. However, there were detectable levels of 1,2,3,4,6,7,8-heptachlorodibenfuran

(1,2,3,4,6,7,8-HpCDF), 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin (1,2,3,4,6,7,8-HpCDD), octachlorodibenzodioxin (OCDD) and the non-ortho-PCB 77 in 6, 10, 9 and 10 supplements, respectively. However, these congeners have low toxic potencies, as reflected by their TEFs. Similar results were reported for three medicinal plants (henna, rosemary, and moghat) with low levels of PCDD/Fs when grown on raw wastewater and analyzed using GC/HRMS (Loutfy et al., 2010). In another study by Mosleh et al. (2014), eight medicinal plants (i.e. caraway, cumin, anise, sage, rosemary, black tea, ginger and cinnamon) randomly collected from Jeddah central market (Saudi Arabia) were investigated using GC-HR/MS, and was concluded that no PCDDs, PCDFs, and dl-PCBs were detected.

To translate the quantified GC-HRMS data into data expressed in toxic equivalents, the concentration of each congener was multiplied by its respective toxic equivalency factor (TEF) and added up to determine the total concentrations in pg TEQ/g of supplement (Van den Berg et al., 2006). It was decided to use for the calculation of the TEQ values the lower bound concentrations, thus, setting values below the LOQ as equal to zero in order to avoid an overestimation of exposure levels (Table 3). The upper bound levels are shown in Table *S2,* and were obtained by setting the concentration in the non-detects equal to the LOQ. Accordingly, the concentrations of PCDD/Fs and dl-PCBs in the samples at the lower bound ranged from 0.01 to 0.19 pg TEQ/g, while the upper bound values ranged from 0.22 to 1.82 pg TEQ/g, with S36 recording the highest lower bound value and S14 the highest upper bound value. Table 3 shows the lower bound concentrations of the 10 supplements, and the corresponding EDI values expressed in pg TEQ/day and in pg TEQ/kg bw per day and per week (assuming daily use). The low amounts of PCDD/Fs and dl-PCBs in the ten supplements corresponded with the observed responses in the DR CALUX® at 48 hrs exposure to extracts treated with an acid silica clean-up, as these samples did not elicit a response (Figure 2b). The ones that did show a low response at 48 hrs with acid-silica, i.e. S10, S14, S29 S36 and S40, were samples that, according to the analytical data, contained some PCDD/Fs plus dl-PCBs, i.e. 0.01, 0.05, 0.08, 0.19 and 0.07 pg TEQ/g, respectively.

Based on the determined PCDD/F and dl-PCB concentrations, the estimated daily intakes (EDIs) (pg TEQ/day) were calculated by multiplying the respective concentration in the supplements with the average weight of the recommended dose and the number of doses to be taken per day. The EDIs ranged from 0.01 to 1.20 pg TEQ/day, where S7 recorded the highest intake level followed by S22 and S25 due to the high recommended daily intakes of the liquid supplements. Intakes per kg bw per day were determined by dividing the EDI (pg WHO-TEQ/day) by the average weight of a consumer. Assuming a daily intake by a 70 kg person, exposure levels ranged from 0.0001 to 0.02 pg TEQ/kg bw/day, which were lower than the exposure levels to PCDD/Fs and dl-PCBs upon food consumption (EFSA 2018). Assuming daily use, the calculated daily exposures translates to a weekly intake of 0.001 to 0.13 pg TEQ/kg bw/week, and are thus substantially lower than the established TWI of 2 pg TEQ/kg bw/week (EFSA, 2018), indicating a low concern for consumers' health.

Table 3. Total PCDD/F and dl-PCB concentrations in the 10 selected samples based on their lower bound values, corresponding EDIs and exposure levels per week, when following the recommended intakes.

				EDI	
Sample ID	PCDD/Fs + dl-PCBs (pg WHO-TEQ /g)	Avg. weight (g)	No. of doses/day	(pg WHO-TEQ/day)	(pg WHO-TEQ/kg bw/week)
S1	0.03	1.07	4	0.13	0.01
S7	0.01	30	4	1.20	0.12
S10	0.01	0.46	2	0.01	0.001
S14	0.05	1.16	4	0.23	0.02
S22	0.01	30	3	0.90	0.09
S25	0.01	60	1	0.60	0.06
S29	0.08	1.09	4	0.35	0.03
S34	0.05	1.34	4	0.27	0.03
S36	0.19	0.96	2	0.36	0.04
S40	0.07	1.14	2	0.16	0.02

The differences in PCDD/F and dl-PCB concentrations (pg WHO TEQ/g) in medicinal plants, is according to Loutfy et al. (2010) likely due to the burden of congeners in different plant locations and differences in the accumulation capacities of the plants. In practice also, processing methods

involving the exposure of products to high temperature and low oxygen may predispose finished products to these unwanted compounds. The evidences thus gathered in the present and these other studies (Foxall et al., 1995; Kim et al., 2000; Fiedler et al., 2002) points at low levels of PCDD/Fs and dl-PCBs in plant based products in contrast to animal derived products (Diletti et al., 2008; Adamse et al., 2017; Hoogenboom et al., 2021). This observation may be explained by the low capability of plants to bioaccumulate these compounds due to their low lipophilicity, unlike foods with high lipid content such as fish, meat and dairy products. Additionally, studies have shown that root uptake and accumulation of PCDD/Fs, dl-PCBs in plants are low, due to their high n-octanol/water partition coefficients (O'Conner et al., 1990; Wild et al., 1992; Webber et al., 1994; Loutfy et al., 2010). Nevertheless, it appeared that for medicinal products, in-field contamination or drying of herbs resulted in a minor risk of introducing high concentrations of dioxins and dl-PCBs.

4.4 Conclusions

This study revealed the successful application of the DR CALUX® bioassay in screening for the presence of less stable and more stable AhR-agonists in a series of selected herbal supplements sold for improved sexual performance, and a confirmation by GC-HRMS of so-called 'suspect samples'. In general, cell responses at 4 and 48 hrs upon exposure to extracts without an acid-silica clean-up can be ascribed to less stable AhR-agonists that were lost upon acid-silica clean-up. Significant amounts of PAHs were detected in some extracts based on the GC-HRMS analysis, however levels of PCDD/Fs and dl-PCBs were low. When comparing the EDIs calculated for the Σ4PAH with the relevant $BMDL_{10}$, the MOE values were far above 10 000, indicating a low health concern and the absence of a need for risk management actions. Similarly, the exposure levels to PCDD/Fs and dl-PCBs were substantially lower than the established TWI of 2 pg TEQ/kg bw/week, thereby also showing low priority for risk management by relevant authorities with respect to the presence of these persistent contaminants in herbal supplements. For further studies, it is recommended to investigate the identity and nature of the unknown natural AhR-agonists in herbal products, that could cause high responses in the DR® CALUX bioassay especially after 48 hrs exposure.

Collaboration

This project is a collaboration between Wageningen Food Safety Research and the Dept. of Toxicology at Wageningen University and Research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY TABLES

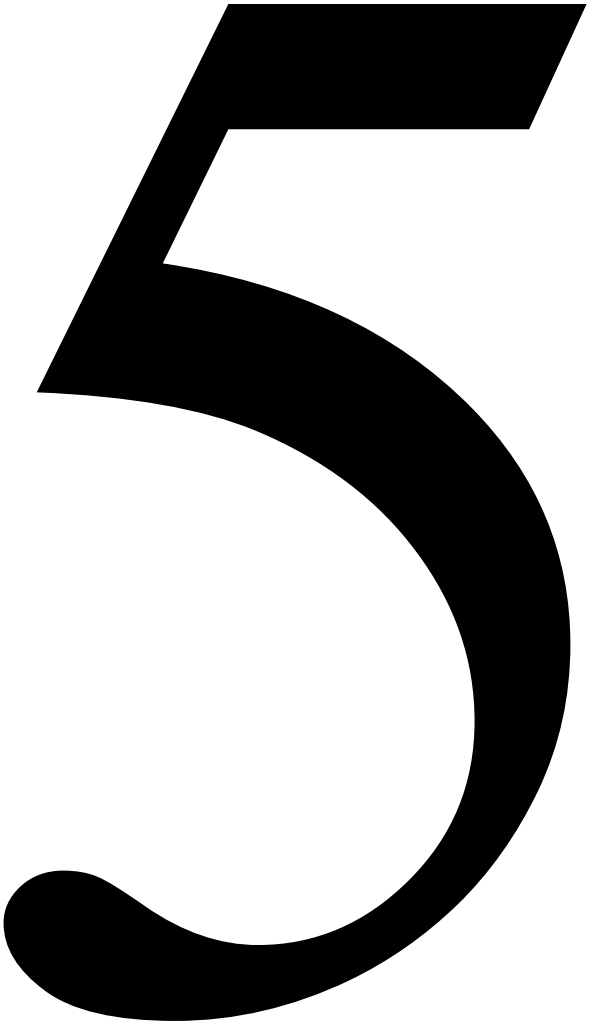
Table S1:List of PAHs detected and estimated concentrations (ng/g) in the GC-HRMS

List PAHs	Concentration in samples (ng/g)									
	S1	S7	S14	S22	S25	S29	S34	S36	S40	S10
Naphthalene	<329	<76.3	<4430	<527	<103	<19.2	<1050	<73.6	<163	<169
Acenaphthylene	<0.1	<0.1	<0.1	<171	<0.1	<4	<0.1	<0.9	<3.4	<3.4
Acenaphthene	<525	<0.1	<3390	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Fluorene	<9.8	<1.2	<0.1	<110	1.83	16.20	<5.3	<4.2	<19.7	<4
Phenanthrene	28.90	2.98	51.80	3.38	3.30	84.60	24.40	24.80	94.30	15.70
Anthracene	<0.3	<0.2	<1.1	<0.3	<0.2	9.65	1.78	1.65	<0.2	2.45
Fluoranthene	20.10	0.64	15.40	0.53	0.64	36.00	8.00	10.80	50.70	8.64
Pyrene	17.00	0.37	12.40	<0.3	<0.4	31.40	6.47	10.10	44.50	7.36
Benzo[c]fluorene	0.68	<0.1	<0.5	<0.1	<0.1	1.19	<0.4	<1.8	<2.2	<0.3
Benzo[a]anthracene	**1.80**	**<0.1**	**<2.3**	**<0.1**	**<0.1**	**7.28**	**<1.6**	**<2.9**	**10.10**	**2.00**
Cyclopenta[cd]pyrene	0.69	<0.2	<2.1	<0.1	<0.2	0.76	<0.4	<0.5	0.69	0.34
Chrysene	**2.83**	**0.11**	**5.97**	**<0.1**	**<0.1**	**11.50**	**<2.6**	**<9.6**	**<12.6**	**2.46**
5-Methylchryseen	<0.1	<0.1	<0.4	<0.1	<0.1	0.30	<0.4	<1.2	<0.2	<0.1
Benzo[b]fluoranthene	**1.11**	**0.14**	**3.65**	**<0.1**	**<0.1**	**3.97**	**2.84**	**3.50**	**6.81**	**2.03**
Benzo[k]fluoranthene	0.31	<0.1	0.64	<0.1	<0.1	1.69	<0.7	<0.3	2.84	0.72
Benzo[j]fluoranthene	0.49	<0.1	1.71	<0.1	<0.1	2.14	<1	0.61	4.43	<1
Benzo[a]pyrene	**0.66**	**<0.1**	**2.83**	**<0.1**	**<0.1**	**2.78**	**1.11**	**1.46**	**5.98**	**1.35**
Indeno[123cd]pyrene	0.43	<0.1	<2	<0.1	<0.1	1.29	1.05	0.74	4.07	0.75
Dibenzo[ah]anthracene	<0.2	<0.1	<1.5	<0.1	<0.1	0.26	<0.2	<0.2	<0.3	0.19
Benzo[ghi]perylene	0.49	<0.1	2.09	<0.1	<0.1	1.43	1.18	0.97	4.64	1.40
Dibenzo[a,l]pyrene	<0.6	<0.2	<4.8	<0.2	<0.2	<0.3	<0.3	<0.4	<2.2	<0.2
Dibenzo[a,e]pyrene	<0.9	<0.2	<10.4	<0.2	<0.2	<0.4	<0.3	<0.5	<0.6	<0.3
Dibenzo[a,i]pyrene	<3.3	<0.9	<42.3	<0.6	<0.8	<1	<0.8	<1.1	<0.7	<0.9
Dibenzo[a,h]pyrene	<3.9	<1	<50.7	<0.8	<0.9	<1.2	<1	<1.3	<0.8	<1.1
Σ_4PAH (lb)	**6.40**	**0.25**	**12.45**	**<0.4**	**<0.4**	**25.53**	**3.95**	**4.96**	**22.89**	**7.84**

Table *S 2:* PCDD/F and dl-PCB concentrations (pg/g) in selected supplements, as determined by GC-HRMS

Analysis series	List of sample										
	WHO-TEF[1]	S1	S7	S14	S22	S25	S29	S34	S36	S40	S10
PCDD/Fs											
2,3,7,8-TCDF	0.1	<0.174	<0.064	<0.534	<0.065	<0.078	<0.164	<0.156	0.21	<0.153	<0.146
1,2,3,7,8-PeCDF	0.03	<0.266	<0.095	<0.462	<0.07	<0.104	<0.296	<0.324	<0.23	<0.19	<0.224
2,3,4,7,8-PeCDF	0.3	<0.26	<0.086	<0.524	<0.068	<0.097	<0.302	<0.306	0.32	<0.178	<0.22
1,2,3,4,7,8-HxCDF	0.1	<0.128	<0.046	<0.238	<0.044	<0.05	<0.122	<0.125	0.14	<0.088	<0.107
1,2,3,6,7,8-HxCDF	0.1	<0.134	<0.048	<0.262	<0.046	<0.052	<0.132	<0.132	0.15	<0.087	<0.107
2,3,4,6,7,8-HxCDF	0.1	<0.148	<0.056	<0.37	<0.051	<0.057	<0.152	<0.145	0.15	<0.103	<0.124
1,2,3,7,8,9-HxCDF	0.1	<0.119	<0.042	<0.238	<0.041	<0.046	<0.111	<0.12	<0.095	<0.081	<0.097
1,2,3,4,6,7,8-HpCDF	0.01	<0.11	<0.032	0.32	0.03	<0.037	1.02	0.14	0.34	0.11	<0.081
1,2,3,4,7,8,9-HpCDF	0.01	<0.176	<0.056	<0.352	<0.039	<0.052	<0.175	<0.151	<0.155	<0.106	<0.138
OCDF	0.0003	<0.326	<0.102	<0.514	<0.108	<0.117	0.652	<0.322	0.50	<0.202	<0.284
2,3,7,8-TCDD	1	<0.164	<0.06	<0.47	<0.058	<0.065	<0.157	<0.192	<0.138	<0.124	<0.154
1,2,3,7,8-PeCDD	1	<0.322	<0.109	<0.758	<0.088	<0.113	<0.306	<0.306	<0.208	<0.258	<0.294
1,2,3,4,7,8-HxCDD	0.1	<0.224	<0.069	<0.396	<0.053	<0.073	<0.212	<0.21	<0.163	<0.141	<0.238
1,2,3,6,7,8-HxCDD	0.1	<0.228	<0.072	<0.424	<0.056	<0.075	<0.224	<0.224	<0.164	<0.15	<0.23
1,2,3,7,8,9-HxCDD	0.1	<0.123	<0.041	<0.238	<0.031	<0.041	<0.119	<0.124	<0.089	<0.082	<0.129
1,2,3,4,6,7,8-HpCDD	0.01	2.64	0.84	4.92	0.88	0.82	5.32	4.00	1.83	3.54	1.34
OCDD	0.0003	0.87	0.32	3.86	0.10	<0.11	65.00	39.00	16.46	21.00	<0.32
PCB 81	0.0003	<0.214	<0.066	<0.364	<0.062	<0.072	<0.18	<0.165	<0.145	0.186	<0.163
PCB 77	0.0001	3.48	0.46	6.14	0.46	0.44	2.32	2.56	2.44	5.42	1.05
PCB 126	0.1	<0.372	<0.114	<0.642	<0.075	<0.096	<0.388	<0.318	<0.262	0.278	<0.266
PCB 169	0.03	<0.396	<0.097	<0.652	<0.087	<0.108	<0.338	<0.274	<0.262	<0.272	<0.228
WHO2005-PCDD/F-PCB-TEQ (lb)		**0.027**	**0.009**	**0.054**	**0.009**	**0.008**	**0.083**	**0.053**	**0.188**	**0.071**	**0.013**
WHO2005-PCDD/F-PCB-TEQ (ub)		**0.784**	**0.266**	**1.821**	**0.227**	**0.280**	**0.823**	**0.822**	**0.630**	**0.613**	**0.689**

[1] Van den Berg et al., 2006



Chapter 5

Determination of pyrrolizidine alkaloids in plant supplements marketed for improved sexual performance and associated health risk to consumers

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In preparation

Abstract

Pyrrolizidine alkaloids (PAs) are a class of secondary plant metabolites that occur in certain plants for defense mechanisms. As a result, their presence in products prepared from these plants is inevitable, albeit that their occurrence could also result from adulteration or cross-contamination with PA producing plants. PAs are noted for their hepatotoxic, genotoxic and carcinogenic effects in animals and humans following metabolic activation in the liver. In this study, herbal supplements sold in Ghana for improving sexual performance were analyzed to determine the presence of 64 PAs using LC-MS/MS analysis.

Up to 17 different PAs were identified in 19 out of the 40 samples. In decreasing order, the most detected PAs were rinderine (in 15 samples), its *N*-oxide (10), intermedine (11), its *N*-oxide (11), lycopsamine (8) and its *N*-oxide (5). The total sum of PAs in samples ranged from 0.005 to 3.2 µg/g, resulting in estimated daily intakes (EDIs) from 0.01 to 12 µg/day, corresponding to exposures from 0.0002 to 0.2 µg/kg bw/day for a 70 kilogram bodyweight person. When compared to a $BMDL_{10}$ of 237 µg/kg bw/day for liver tumours in rats treated with riddelliine, the margin of exposure (MOE) ranged from 1200 to 1,400,000 with 8 samples showing MOE values below 10 000, thus indicating a health concern.

5.1 Introduction

Pyrrolizidine alkaloids (PAs) are heterocyclic organic secondary metabolites produced by plants for defensive roles (Ober and Kaltenegger, 2009). They are found in certain plant families including Apocynaceae, Asteraceae (Compositeae), Boraginaceae, Leguminosae (Fabaceae), Orchidaceae, Ranunculaceae, and Scrophulariaceae (Fu et al. 2002; Gerardo, 2005; Wiedenfield, 2014). PAs occur in botanical preparations when PA containing raw materials are used, or when products are contaminated and or adulterated with PA producing plants during harvesting or processing of raw materials. PAs may occur in botanicals as tertiary amine (free base) or in their *N*-oxide form (i.e. PA-*N*-oxide or PANO) (Hartmann and Witte, 1995; Langel et al. 2011; Gottschalk et al. 2019). Thus, more than 660 different PAs and their *N*-oxides have been associated with over 6000 plant species and more than 3 % of flowering plants (Smith et al. 1981). Information on their structures, classifications and metabolism has been extensively discussed in other studies and reviews (Stegelmeier et al. 1999; Pereira et al. 2018; Dusemund et al. 2018; Hessel-Pras et al. 2019; Chen et al. 2019).

Unlike saturated PAs (platynecine), which are non-genotoxic, 1,2-unsaturated PAs (e.g. riddelliine, lasiocarpine and monocrotaline) are known genotoxic carcinogens (EFSA, 2017) due to their metabolic activation in the liver by cytochrome P450 monooxygenases, resulting in the formation of highly reactive electrophilic pyrrolic intermediates (Mattocks 1968; Culvenor et al. 1969; Dusemund et al. 2018). These metabolites (alkylating agents) once formed, can bind to endogenous nucleophiles such as proteins and nucleic acids, resulting in cell damage, cell death and/or induction of tumour formation (Mattocks, 1986; Winter and Segall, 1989; Fu et al. 2002; Gottchalk et al. 2018). Furthermore, because PA-*N*-oxides can be reduced to their corresponding tertiary amines (free base) in the gut and liver, they are equally considered toxic (Mattocks, 1971; Gottchalk et al. 2018). PA toxicity depends on the alkaloid, dosage, nature and duration of exposure, individual susceptibility, rate of bioactivation to pyrroles and chemical reactivity of the pyrrole produced (Mattocks 1986; Roeder, E. 1995; Stegelmeier et al. 1999; Azzreena et al. 2019).

The Scientific Panel on Contaminants in the Food Chain (CONTAM Panel) of the European Food Safety Authority (EFSA) has indicated that exposure to PAs in herbal dietary products, could present both acute and chronic effects in consumers. Acute human exposure to toxic PAs is characterized by hepatic sinusoidal obstruction syndrome (HSOS) which may likely induce severe liver injury and lead to liver cirrhosis and failure. Chronic exposures may lead to abnormalities in the liver and lungs, including tumours and the development of pulmonary arterial hypertension (Fu et al. 2002; Gerardo, 2005; Wiedenfeld, 2011; EFSA, 2011; Li et al. 2018).

Studies on PA contaminated tea, honey, herbal infusions, medicines and dietary supplements have been reported in different countries including Ghana (Kakar et al. 2010; Letsyo et al. 2017; Chen et al. 2019; Suparmi et al. 2020). Letsyo et al. (2017) carried out studies explicitly focused on honey and a selected number of herbal medicinal products in Ghana. However, there is still a lack of relevant data on the presence of PAs in most herbal products/supplements currently flooding the Ghanaian market. The present study is part of a holistic approach to investigate plant supplements sold on the Ghanaian market for improving sexual performance. In a previous study by Akuamoa and others (2021), the inhibition potentials of these supplements were determined using the PDE-Glo bioassay. A follow-up study was conducted using a tiered approach involving a combination of biological and chemical analysis to investigate if the observed inhibition potentials were inherent plant constituents or well-known synthetic PDE-5 inhibitors intentionally added by producers to enhance expected outcomes (unpublished). Subsequently, the presence of polycyclic aromatic hydrocarbons (PAHs) and dioxins and dioxin-like polychlorinated biphenyls were determined due to the potential genotoxic, carcinogenic and endocrine disruptive effects of these compounds in humans (unpublished). The objective of the current study was to analyze the selected samples for the potential presence of PAs. Based on the results, a cumulative risk assessment of total PAs was performed using recommended daily intakes of the respective botanical preparations to estimate exposure levels of consumers and resulting margins of exposure (MOE).

5.2 Materials and methods

5.2.1 Supplements

The forty herbal supplements previously examined by Akuamoa and others (2021) were further investigated in this study (refer to *TS1* of the previous study for the list of supplements, their corresponding IDs, country of origin, composition, intended use, directions for use, and other relevant information). The criteria for sample selection were mainly based on their intended use (i.e. for improved sexual performance). In all, the selected samples included 9 liquids and 31 capsules. The contents of each capsule were poured into polyethylene zip lock bags and mixed, while liquid-based supplements were manually agitated for five minutes prior to preparation.

5.2.2 Chemicals and reagents

A total of 64 PA standards were used in this analysis. Details on vendors, purity and CAS numbers can be found in Table S1. The purity of the standards was at least 95%. Spartioidine *N*-oxide was synthesized in-house following the method of Chou et al. (2003). PA stock solutions of 200 µg/mL were prepared in methanol. A mixed PA solution of 1 µg/mL in methanol prepared from each stock solution was used to spike supplements. Methanol and acetonitrile both of LC-MS grade were obtained from Actu-All (Oss, The Netherlands). Both formic acid and ammonium carbonate were of analytical grade and purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands). Deionized water (MilliQ) with a minimum resistance of 18.2 M was used as a solvent.

5.2.3 Sample extraction and purification

Sample extraction was performed based on an in-house validated method as described by Chen et al. (2019). Briefly, 1 g or mL of solid or liquid sample was either weighed or aliquoted in duplicate into extraction tubes. First, one of the test samples was spiked with 0.25 µg PAs/g by adding 250 µL mixed PA standard solution. To the tubes 20 mL of 0.2% formic acid solution in water was added and the samples were agitated in a rotary tumbler for 30 min and subsequently centrifuged for 15 min at 3500 *g*. Next, 5 mL of each sample extract was aliquoted into new tubes, where the extracts were neutralized to a pH of 6-8 with 350 µL of 1 M ammonium carbonate solution and subsequently centrifuged at 3500 g for another 15 min.

Each extract was purified using solid-phase extraction (SPE) (Strata-X Polymeric reversed-phase 200 mg/6 mL cartridges, Phenomenex, Palo Alto, CA, USA). Cartridges were pre-conditioned with 6 mL methanol, followed by 6 mL water. The whole content of each neutralized extract was passed through individual cartridges, and subsequently washed with 6 mL 1% formic acid, followed by 6 mL water. Cartridges were dried for 10 min under reduced pressure using an SPE vacuum manifold. Next, the compounds of interest were eluted with 6 mL of methanol. Eluates were subsequently dried under a continuous stream of nitrogen at 50 °C using a TurboVap (Biotage, Uppsala, Sweden). Resultant residues were filtered through 0.45 µm PTFE filter vials (UniPrep, Whatman, Maidstone, UK) after reconstitution with 500 µL methanol/water 10/90, v/v. Vials were closed using a compressor, and stored at -20 °C until analysis.

5.2.4 LC-MS/MS analysis

The LC-MS/MS system used consisted of a Waters Acquity UPLC coupled to a Xevo TQ-S tandem mass spectrometer (Waters, Milford, MA, USA). Chromatographic separation of analytes was carried out on a UPLC BEH C18 analytical column (Waters, Milford, MA, USA) with 150 × 2.1 mm, 1.7 µm particle size, with column and sample temperature set at 50 °C and 10 °C, respectively. The analytes were eluted with a gradient program consisting of two mobile phases: water containing 10 mM ammonium carbonate pH 9 (A) and acetonitrile (B) and a flow rate of 0.4 mL/min. The linear gradient started at 0 % B, increased to 5 % B at 0.1 min, ~~to~~ 10 % B at 3.0 min, to 24 % B at 7.0 min, and 70 % B at 12.0 min. Finally, B was decreased to 0 % at 12.1 min and kept at this composition for 2.1 min. The injection volume was 2 µL for each sample extract.

The linearity of the LC-MS/MS was assessed using matrix matched standards (MMS) (8 points) to confirm the accuracy of the sample pre-treatment. For this, 8 test samples (1 g) of a previously tested supplement with no indication of PAs (<LOD) were spiked with a mixture of the PA standards in a concentration range of 0 to 1.0 µg/g. The MMS were treated and analyzed following the procedure described above. The LOQ was 0.005 µg/g for individual PAs in solid samples and 0.005 µg/mL in liquids.

PAs in samples were detected and confirmed by comparing their retention times and ion ratios with those of the calibration curves of compounds in the MMS (using two MRM transitions measured per analyte). In Table *S2,* the mass spectrometric settings for each analyte are given. Individual sample concentrations were determined based on each sample's single level standard addition (0.25 µg/g). Data were processed using the TargetLynx 4.2 software (Waters Corporation, Milford, MA, USA).

5.2.5 Calculation of estimated daily intake (EDI)

The estimated daily intake (EDI) expressed as µg/day was calculated as follows:

i. $EDI = Total\ PAs\ x\ Average\ weight\ x\ No.of\ \ doses$

where :

- Total PAs is the total concentration of the PAs detected in each sample by LC-MS/MS, expressed as µg/g (solid samples) or µg/mL (liquid samples).
- Average weight is the average weight per dose unit expressed in gram or mL.
- No. of doses is the recommended number of doses per day.
- The estimated daily intake (EDI) expressed as µg/kg bw/day was calculated as follows:

ii. $\text{EDI} = \frac{EDI\ (\mu g/day)}{BW\ (kg)}$

where :

- EDI is the calculated estimated daily intake based on the total PA concentration, the average dose and the number of recommended doses per day.
- BW is the average body weight of consumers (mostly males), assumed to be 70 kg.

5.2.6 Risk characterization based on the EDI

The margin of exposure (MOE) approach was used for risk characterization of 1,2-unsaturated PAs in samples due to their genotoxic and carcinogenic properties as recommended by the European Food Safety Authority (EFSA, 2005). To assess the potential risk of PAs as a result of chronic exposure, EFSA established a benchmark dose ($BMDL_{10}$) of 237 µg/kg bw/day, which is the lower bound of the dose that showed a 10 % increase in the incidence of liver tumors in rats treated with riddelliine (NTP 2003), as the reference point for calculating the margin of exposure (MOE) (EFSA, 2017).

- MOE : $\frac{BMDL10}{EDI}$

For genotoxic carcinogens, EFSA (2005) considers an MOE larger than 10 000 as indicative of a low health concern.

5.3 Results and discussion

The 40 supplements were analyzed for the presence of 64 PAs. Overall, 19 (48 %) out of the 40 contained 17 different PAs (10 free bases and 7 *N*-oxides) (Table 1). The sum of PAs in their free bases ranged from 0.005 to 3.2 µg/g, whereas the *N*-oxides ranged from 0.01 to 1.3 µg/g. Overall, the total PAs (i.e. the sum of free bases plus *N*-oxides) ranged from 0.005 to 3.2 µg/g. S12 contained the highest levels (2.5 µg/g) of free bases followed by S38 (1.5 µg/g). However, considering *N*-oxides, the sample with the highest concentration was S27 (1.3 µg/g) followed by S26 (0.8 µg/g). S12 (3.2 µg/g) contained the highest amounts of total PAs followed by S27 (1.8 µg/g). The average of the total PA was higher than the amounts detected in other Ghanaian medicinal plants (Letsyo et al. 2017). Overall, the PA levels in 8 samples were above the maximum established level for supplements (i.e. 400 µg/kg or 0.4 µg/g), by the European Commission (EC, 2020).

The identified PAs in all 19 samples were open chained monoesters and open chained diesters of retronecine- and heliotridine-type necine bases. Figure 1 groups samples with similar PA profiles based on their relative contributions to the total PA. Nine samples had a combination of rinderine, intermedine, lycopsamine and their *N*-oxides (Fig. 1a). Two samples (S23, S38) had a combination of europine, heliotrine and lasiocarpine, with traces of heliotrine *N*-oxide and rinderine (Fig. 1b). Two other samples (S26, S28) contained primarily indicine and its N-oxide, with traces of rinderine, intermedine and its *N*-oxide (Fig. 1c). Finally, one sample (S12) contained predominantly echinatine and its *N*-oxide together with traces of rinderine, intermedine, lycopsamine and their *N*-oxides (Fig. 1d).

One crucial question is whether the source of PAs in the supplements could be related to one or more of the plant ingredients mentioned on their labels. A total of 72 different ingredients were indicated on the labels of the 40 samples. Interestingly, only one ingredient, *Heliotropium indicum*, is a known PA-producing plant (El-Shazly & Wink, 2014). This plant was also mentioned by Letsyo et al. (2017) as an ingredient of medicinal plants in Ghana. A study by Singh et al. (2005) on *H. indicum* pointed at the presence of indicine, heliotrine, lycopsamine, echinatine and lasiocarpine. Roeder and

Wiedenfeld (2009) addressed the hepatoxic effects of alkaloids in *H. indicum* and discouraged their oral intake. Although this plant was mentioned on the label of S39, this supplement only contained trace amounts of rinderine.

Six samples (S1, S12, S17, S20, S23, S39) contained plants (*Pueraria tuberosa, Abrus precatorius, Dalbergia saxatilis*, *Astragalus root, Mucuna puriens*) belonging to the Fabaceae plant family. However, none of these plants is known to contain PAs. Three other samples (S6, S21, S38) produced from the same plant materials (*Penianthus zenkeri, Clausena anisata)*, contained different amounts of PAs. In two of these samples (S6, S21), four similar PAs (intermedine, rinderine and their *N*-oxides) were identified, while the PAs identified in S38 were different (Table 1). Combined with the relatively low PA levels, these observations point to the possibility of contamination with PA-containing plants during production.

Table 1: Identified PAs (free bases and *N*-oxides) and their respective total concentrations (µg/g) in positive samples

List of samples	7-Acetylintermedine	7-Acetylintermedine N-ox	Echinatine	Echinatine N-ox	Europine	Heliotrine	Heliotrine N-ox	Indicine	Indicine N-ox	Intermedine	Intermedine N-ox	Lasiocarpine	Lycopsamine	Lycopsamine N-ox	Monocrotaline	Rinderine	Rinderine N-ox	Sum Free Bases (µg/g)	Sum *N*-oxides (µg/g)	Sum 60 PAs (µg/g)
S2										0.19	0.24		0.01	0.01		0.16	0.39	0.36	0.64	1.00
S5										0.04	0.06					0.05	0.10	0.09	0.15	0.24
S6										0.20	0.20		0.01	0.01		0.17	0.40	0.38	0.61	0.99
S8										0.11	0.04		0.01			0.15	0.14	0.26	0.18	0.44
S9			0.01															0.01		0.01
S12			2.37	0.61						0.01	0.04		0.02	0.01		0.12	0.03	2.51	0.69	3.20
S16								0.01	0.01	0.01	0.02					0.02	0.05	0.04	0.08	0.12
S17													0.01					0.01		0.01
S18																0.01		0.01		0.01
S21										0.08	0.13		0.02	0.01		0.07	0.16	0.17	0.30	0.47
S23					0.01	0.04												0.05		0.05
S26			0.01					0.12	0.80	0.01	0.02							0.13	0.83	0.96
S27	0.01	0.01		0.01						0.27	0.39		0.05	0.05	0.01	0.26	0.81	0.59	1.27	1.86
S28								0.02	0.09							0.01		0.03	0.09	0.11
S29																0.01		0.01		0.01
S30										0.02	0.02		0.01			0.01	0.02	0.04	0.04	0.08
S37										0.01	0.01					0.01	0.02	0.02	0.03	0.05
S38			0.01		0.18	1.20	0.01					0.13				0.02		1.54	0.01	1.55
S39																0.01		0.01		0.01

Figure 1(a)

Figure 1(b)

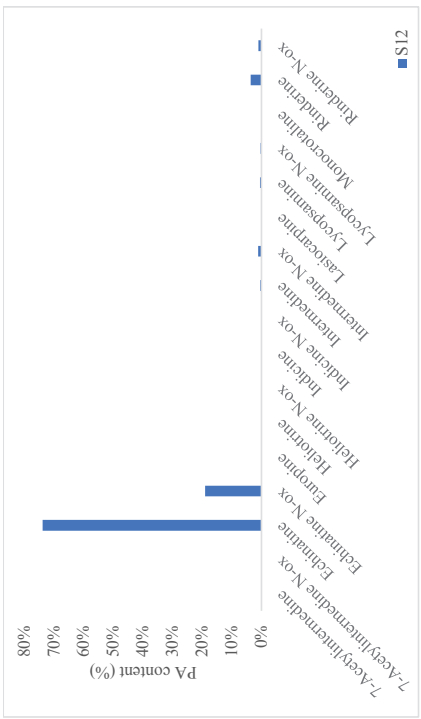

Figure 1(d)

Figure 1(c)

Fig. 1 PA profiles found in samples, grouped according to similarity. Fig. 1 (a) nine samples with primarily intermedine, rinderine and their *N*-oxides (b), two samples with europine and heliotrine, (c) two samples with indicine and its *N*-oxide, (d) one sample with echinatine and its *N*-oxide

The estimated daily intakes (EDI) (µg/day) and intakes per kilogram body weight per day (µg/kg bw/day) was calculated based on the formulas *(i)* and *(ii)* respectively, of section 5.2.5 (Table 2). The EDIs ranged from 0.01 to 12.42 µg/day, which resulted in intakes ranging from 0.0002 to 0.19 µg/kg bw/day, with S9 and S27 showing the lowest and highest exposures, respectively. Generally, samples with higher concentrations showed higher intake levels; however, some samples with relatively low concentrations also resulted in relatively higher intakes due to the high recommended intakes per day (e.g. S30).

Table 2: Levels of PAs in positive samples, their respective recommended no. of doses per day and resulting EDIs, and MOEs.

Sample	ΣPAs (µg/g)	Average dose (g)	No. of doses per day	EDI (µg/day)	EDI (µg/kg bw/day)	MOE
S2	1.0	0.81	4	3.24	0.05	4,800
S5	0.24	1.01	4	0.97	0.01	16,000
S6	0.99	0.47	2	0.93	0.01	17,000
S8	0.44	2.01	6	5.33	0.08	2,900
S9	0.01	0.51	2	0.01	0.0002	1,420,000
S12	3.20	0.74	2	4.74	0.07	3,300
S16	0.12	0.65	2	0.16	0.002	98,000
S17	0.01	1.10	4	0.03	0.0005	500,000
S18	0.01	0.65	4	0.02	0.0003	894,000
S21	0.47	0.97	4	1.82	0.03	8,500
S23	0.05	20	1	1.08	0.02	14,000
S26	0.96	1.10	2	2.10	0.03	7,300
S27	2.00	1.67	4	12.42	0.19	1,200
S28	0.11	2.13	6	1.46	0.02	10,600
S29	0.01	1.09	4	0.02	0.0003	740,000
S30	0.10	30	4	9.79	0.15	1,600
S37	0.05	2.03	4	0.39	0.01	39,700
S38	1.55	1.47	4	9.13	0.14	1,690
S39	0.01	1.70	4	0.06	0.001	240,500

The calculated intakes per kg bw/day were all below 10 µg/kg bw/day, which is the concentration likely to cause hepatic veno-occlusive disease (HVOD) in humans (WHO-IPCS, 1988; EFSA 2016). However, the International Agency for Research on Cancer (IARC, 1976) has classified certain PAs such as lasiocarpine and monocrotaline as possibly carcinogenic to humans (Group 2B). Furthermore, due to the genotoxic and carcinogenic adverse effects of 1,2 unsaturated PAs in general, the EFSA concluded that no safe threshold value can be derived. Nonetheless, due to their unavoidable nature, the margin of exposure (MOE) approach is best suited when characterizing the risk of upon exposure to products containing toxic PAs. Based on the estimated intakes, the MOE values were determined according to the formula in 2.6, using the $BMDL_{10}$ derived by EFSA for liver tumors in rats exposed to riddelliine. As shown in Table 2, the calculated MOE values ranged from 1200 to 1,400,000, where 8 samples had MOE values below 10,000. It should be noted that in general, this approach assumes life-long intake of a supplement likely to present a worst case scenario. It should also be noted that, in the study with lasiocarpine (NTP, 1978), many animals died before the end of the study due to ruptures of blood vessels in the liver caused by the haemangiosarcomas. This implies that there is no need for long-term exposure to necessarily induce adverse outcomes.

In the above calculations, the various PAs detected in the samples were assumed equal potencies. However, in practice there are strong indications that most PAs are less toxic than riddelliine for which the reference points were derived. Merz and Schrenk (2016) evaluated available information and proposed provisional relative potency factors (RPFs) for a number of PAs. Similar RPFs were also established by Louise et al. (2019) based on genotoxic potencies of PAs in the human liver cell-line (HepaRG). The RPFs for the different PAs detected in the supplements varied between 0.01 and 1. These RPFs were applied on the levels of each PA, as shown in Table *S3*. PA-*N*-oxides were assumed the same potency as that of their free bases. The resulting equivalent levels for the sum of PAs varied between 0.0001 and 0.94 µg/g. Still, the S12 contained the highest sum of PAs (0.94 µg/g) based on the RPF of the identified PAs (i.e. echinatine, intermedine, lycopsamine, rinderine and their *N*-oxides). Using these levels expressed in riddelliine equivalents, EDIs decreased by 2.8 to 100,

while the MOE values increased by the same factors. Nevertheless, the MOE values of S27 and S38 were still lower than 10 000, thus indicating a health concern in principle upon life-long exposure (Table *S3*). This clearly shows that, apart from toxic PAs (indicating a health concern), the type of PA may equally play a significant role in the magnitude or risk for using these supplements. Nevertheless, at the moment, there is no international consensus about the magnitude and use of the proposed RPFs in risk assessment or risk management of toxic PAs in botanical preparations. There is also no generally accepted method to correct for a shorter exposure duration. While this relatively small study points at the possible contamination of these supplements with toxic PAs, it remains unclear if higher contamination levels may occur in herbal preparations intentionally prepared using PA containing herbal ingredients.

5.4 Conclusions

This study is part of a holistic approach in assessing the safety of plant supplements sold in Accra for improving sexual performance. The current objective was to analyze the selected supplements for the potential presence of PAs to assess the possible risk for users upon exposure. The study revealed the presence of PAs in almost 50 % of the supplements, which did not correlate to the ingredients listed on their labels in almost all cases. The relatively low levels determined implied that the source is likely related to contamination of ingredients with PA containing plants. Whilst the estimated intake levels were generally low, exposure levels for eight samples pointed at a potential health concern. Considering differences in toxic potencies of PAs, only two samples had MOE values below 10 000, thus indicating a concern.

Collaboration

This project is a collaboration between Wageningen Food Safety Research and the Dept. of Toxicology at Wageningen University and Research.

Declaration of Competing Interest

The authors declare that they have no known competing financial interest or personal relationship that could have appeared to influence the work reported in this paper.

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SUPPLEMENTARY LIST

Table *S1*: List of 64 PA standards used in analysis. Details on vendors, purity and CAS numbers

Pyrrolizidine alkaloid	CAS no.	Supplier	Article code	Purity (%)
7-O-Acetylintermedine	74243-01-9	PhytoPlan	6276.95	>95
7-O-Acetylintermedine *N*-oxide	685132-59-6	PhytoPlan	6277.95	>95
7-O-acetyllycopsamine (hydrochloride)	73544-48-6	PhytoPlan	6272.95	>95
7-O-Acetyllycopsamine *N*-oxide	685132-58-5	PhytoPlan	6273.97	>97
Echimidine	520-68-3	PhytoPlan	6278.95	97.78
Echimidine *N*-oxide	41093-89-4	PhytoPlan	6279.97	>97
Echinatine	480-83-1	PhytoPlan	6295.95	>95
Echinatine *N*-oxide	202067-93-0	PhytoPlan	6296.95	>95
Echiumine	633-16-9	PhytoLab	83749	100
Echiumine *N*-oxide	685554-68-1	PhytoLab	84164	95.1
Erucifoline	40158-95-0	PhytoPlan	6218.97	99.91
Erucifoline *N*-oxide	123864-94-0	PhytoPlan	6221.97	99.6
Europine (hydrochloride)	570-19-4	PhytoPlan	6214.97	>97
Europine *N*-oxide	65582-53-8	PhytoPlan	6215.97	99.73
Heliosupine	32728-78-2	PhytoPlan	6297.95	>95
Heliosupine *N*-oxide	31701-88-9	PhytoPlan	6297.95	>95
Heliotrine	303-33-3	PhytoPlan	6212.98	98.96
Heliotrine *N*-oxide	6209-65-0	PhytoPlan	6213.97	>97
Indicine (hydrochloride)	1195140-94-3	PhytoLab	83234	98.9
Indicine *N*-oxide	41708-76-3	PhytoPlan	6217.96	>96
Integerrimine	480-79-5	PhytoPlan	6283.97	>97
Integerrimine *N*-oxide	85955-28-8	PhytoPlan	6284.95	>95
Intermedine	10285-06-0	PhytoPlan	6274.95	>95
Intermedine *N*-oxide	95462-14-9	PhytoPlan	6275.95	>95
Jacobine	6870-67-3	PhytoPlan	6219.98	>98
Jacobine *N*-oxide	38710-25-7	PhytoPlan	6222.96	96.45
Jacoline	480-76-2	PhytoPlan	6291.97	>97
Jacoline *N*-oxide	1148039-73-9	PhytoPlan	6292.97	>97
Jaconine	480-75-1	PhytoPlan	6293.95	>95
Jaconine *N*-oxide	1148039-75-1	PhytoPlan	6294.95	>95
Junceine	480-53-5	PhytoLab	85643	100
Junceine *N*-oxide		PhytoLab	85305	99.86
Lasiocarpine	303-34-4	PhytoPlan	6210.97	>97
Lasiocarpine *N*-oxide	127-30-0	PhytoPlan	6211.96	96.74
Lycopsamine	10285-07-1	PhytoPlan	6270.95	97.45
Lycopsamine *N*-oxide	95462-15-0	PhytoPlan	6271.95	98.74
Merenskine	96657-94-2	PhytoPlan	6223.97	>97
Merenskine *N*-oxide	96657-95-3	PhytoPlan	6225.97	99.53
Merepoxine	115777-94-1	PhytoPlan	6224.97	99.93
Merepoxine *N*-oxide		PhytoPlan	6226.97	98.23
Monocrotaline	315-22-0	PhytoPlan	6227.99	99.25
Monocrotaline *N*-oxide	35337-98-5	PhytoPlan	6228.98	99.94

Otosenine	16958-29-5	PhytoLab	80879	>95
Retrorsine	480-54-6	PhytoPlan	6203.98	>98
Retrorsine *N*-oxide	15503-86-3	PhytoPlan	6253.96	>96
Riddelliine	23246-96-0	PhytoPlan	6312.98	>98
Riddelliine *N*-oxide	75056-11-0	PhytoPlan	6313.97	>97
Rinderine	6029-84-1	PhytoPlan	6310.95	>95
Rinderine *N*-oxide	137821-16-0	PhytoPlan	6311.95	>95
Sceleratine	6190-26-6	PhytoPlan	6229.97	>97
Sceleratine *N*-oxide	103184-92-5	PhytoPlan	6230.97	>97
Senecionine	130-01-8	PhytoPlan	6202.99	>99
Senecionine *N*-oxide	13268-67-2	PhytoPlan	6252.95	>95
Seneciphylline	480-81-9	PhytoLab	89275	99.8
Seneciphylline *N*-oxide	38710-26-8	PhytoLab	82632	99.6
Seneciverinine	72755-25-0	PhytoPlan	6206.95	98.95
Senecivernine *N*-oxide	101687-28-9	PhytoPlan	6220.95	99.47
Senkirkine	2318-18-5	PhytoPlan	6205.95	98.01
Spartioidine	520-59-2	PhytoPlan	6314.95	95.1
Spartioidine *N*-oxide	121123-61-3	in house		
Trichodesmine	548-90-3	PhytoLab	83438	99.61
Trichodesmine *N*-oxide	55727-46-3	PhytoLab	84738	100
Usaramine	15503-87-4	PhytoPlan	6315.96	>96
Usaramine *N*-oxide	117020-54-9	PhytoPlan	6316.96	>96

PhytoLab: Vestenbergsgreuth, Germany

PhytoPlan: Heidelberg, Germany

Table *S2*: Mass spectrometric settings for each compound in the reference library

Pyrrolizidine alkaloid	Precursor ion (m/z)	Cone voltage (V)	Product ion 1 (m/z)	Col. energy 1 (eV)	Product ion 2 (m/z)	Col. energy 2 (eV)	Product ion 3 (m/z)	Col. energy 3 (eV)	Indicative RT (min)	MRM window
7-O-Acetylintermedine	342.2	30	120	30	180	20	94	40	8.80[a]	4
7-O-Acetylintermedine *N*-oxide	358.2	30	137	30	214	30	180	30	5.90[b]	5
7-O-acetyllycopsamine	342.2	30	120	30	180	20	94	40	8.85[a]	4
7-O-Acetyllycopsamine *N*-oxide	358.2	30	137	30	214	30	180	30	5.90[b]	5
Echimidine	398.2	30	120	25	220	20	83	25	10.55	6
Echimidine *N*-oxide	414.2	30	254	30	352	25	94	40	7.40	4
Echinatine	300.2	30	138	30	156	30	94	35	6.45	4
Echinatine *N*-oxide	316.2	30	111	40	172	30	94	40	3.75	5
Echiumine	382.2	30	120	45	83	35	94	35	11.45	6
Echiumine *N*-oxide	398.2	30	220	25	94	40	83	40	9.15	6
Erucifoline	350.2	40	94	40	120	30	138	30	7.30	1
Erucifoline *N*-oxide	366.2	40	94	40	118	30	120	30	3.25	2
Europine	330.2	30	94	35	138	30	156	30	6.15	5
Europine *N*-oxide	346.2	30	172	30	111	40	256	25	3.55	5
Heliosupine	398.2	30	120	25	220	20	336	20	10.40	6
Heliosupine *N*-oxide	414.2	30	94	30	254	30	138	30	7.10	4
Heliotrine	314.2	30	138	25	156	25	94	35	8.10	4
Heliotrine *N*-oxide	330.2	30	111	35	172	25	94	40	5.50	5
Indicine	300.2	30	94	35	156	30	138	30	5.50[c]	4
Indicine *N*-oxide	316.2	30	94	40	172	30	111	40	3.55	5
Integerrimine	336.2	40	94	40	120	30	138	30	9.95	3
Integerrimine *N*-oxide	352.2	40	94	40	120	30	136	30	6.50	1
Intermedine	300.2	30	94	35	156	30	138	30	5.45[c]	4
Intermedine *N*-oxide	316.2	30	94	40	172	30	111	40	3.40[d]	5
Jacobine	352.2	40	120	30	155	30	94	40	7.65	1
Jacobine *N*-oxide	368.2	40	120	30	296	25	119	30	4.40	2
Jacoline	370.2	40	94	40	138	30	120	30	5.35	2

Jacoline *N*-oxide	386.2	40	94	40	120	30	138	30	2.60	1
Jaconine	388.2	40	94	40	120	30	138	30	8.75	3
Jaconine *N*-oxide	404.2	40	94	40	120	35	118	35	4.95	1
Junceine	370.2	40	222	30	120	30			7.50	3
Junceine *N*-oxide	386.2	40	94	40	120	30	138	30	4.60	2
Lasiocarpine	412.2	30	120	25	220	20	336	20	11.25	6
Lasiocarpine *N*-oxide	428.2	30	138	30	254	25	94	40	8.25	6
Lycopsamine	300.2	30	94	35	156	30	138	30	5.50[c]	4
Lycopsamine *N*-oxide	316.2	30	94	40	172	30	111	40	3.45[d]	5
Merenskine	388.2	40	94	35	120	30	138	30	9.05	3
Merenskine N-oxide	404.2	40	94	40	120	35	118	35	5.10	1
Merepoxine	352.2	40	94	40	120	30	138	30	8.25[e]	1
Merepoxine *N*-oxide	368.2	40	94	40	120	30	119	30	5.00	2
Monocrotaline	326.2	40	94	35	120	30	121	30	5.60	2
Monocrotaline *N*-oxide	342.2	40	120	35	137	30	94	40	2.45	2
Otosenine	382.2	30	122	30	168	25	150	25	4.55	2
Retrorsine	352.2	40	94	40	120	30	138	30	8.55	1
Retrorsine *N*-oxide	368.2	40	94	40	120	30	119	30	5.40	2
Riddelliine	350.2	40	94	40	120	30	138	30	7.80	1
Riddelliine *N*-oxide	366.2	40	94	40	118	30	120	30	4.50	2
Rinderine	300.2	30	138	30	156	30	94	35	6.60	4
Rinderine *N*-oxide	316.2	30	111	40	172	30	94	40	3.85	5
Sceleratine	370.2	40	94	40	138	30	120	30	5.75	2
Sceleratine *N*-oxide	386.2	40	94	40	120	30	138	30	2.80	1
Senecionine	336.2	40	94	40	120	30	138	30	10.25	3
Senecionine *N*-oxide	352.2	40	94	40	120	30	136	30	6.70[f]	1
Seneciphylline	334.2	40	120	30	138	30	94	40	9.25	3
Seneciphylline *N*-oxide	350.2	40	94	40	138	30	118	30	5.80	1
Seneciverinine	336.2	40	94	40	120	30	138	30	10.40	3
Senecivernine *N*-oxide	352.2	40	94	40	120	30	136	30	6.75[f]	1

Senkirkine	366.2	30	122	30	168	25	150	25	7.00	1
Spartioidine	334.2	40	120	30	138	30	94	40	8.95	3
Spartioidine *N*-oxide	350.2	40	94	40	120	30	118	30	5.70	1
Trichodesmine	354.2	40	120	35	222	30	121	30	8.65	1
Trichodesmine *N*-oxide	370.2	40	137	30	238	30	94	40	5.30	2
Usaramine	352.2	40	94	40	120	30	138	30	8.30[e]	1
Usaramine *N*-oxide	368.2	40	94	40	120	30	119	30	5.25	2

Table *S3*: List of samples with identified PAs, their necine bases and relative potencies. PA N-oxides were assumed to have the same potency as their free bases.

List of samples	7-Acetylintermedine	7-Acetylintermedine N-ox	Echinatine	Echinatine N-ox	Europine	Heliotrine	Heliotrine N-ox	Indicine	Indicine N-ox	Intermedine	Intermedine N-ox	Lasiocarpine	Lycopsamine	Lycopsamine N-ox	Monocrotaline	Rinderine	Rinderine N-ox	Σ64 PAs based on RPF (µg/g)	EDIs based on RPFs (ug/g)	EDIs ug/kgbw/day)	MOE based on RPFs
Necin	R	R	H	H	H	H	H	R	R	R	R	H	R	R	R	H	H				
RPF	0.1	0.01	0.3	0.3	0.3	0.3	0.3	0.01	0.01	0.01	0.01	1	0.01	0.01	1	0.3	0.3				
S2										0.002	0.002		0.0001	0.0001		0.05	0.12	0.17	0.55	0.01	27900
S5										0.0004	0.001					0.015	0.03	0.04	0.18	0.00	87400
S6										0.002	0.002		0.0001	0.0001		0.05	0.12	0.18	0.16	0.00	93900
S8										0.001	0.0004		0.0001			0.044	0.04	0.09	1.05	0.02	14700
S9			0.003															0.003	0.00	0.00	4727000
S12			0.71	0.18						0.0001	0.0004		0.0002	0.0001		0.04	0.010	0.94	1.39	0.02	11100
S16								0.0001	0.0001	0.0001	0.0002					0.01	0.015	0.02	0.03	0.00	537300
S17													0.0001					0.0001	0.00	0.00	49550000
S18																0.002		0.002	0.01	0.00	2979000
S21										0.001	0.001		0.0002	0.0001		0.02	0.05	0.07	0.28	0.00	55900
S23					0.003	0.01												0.02	0.33	0.01	47400
S26			0.001					0.001	0.01									0.01	0.02	0.00	641000
S27	0.001	0.0001		0.002						0.003	0.004		0.0005	0.0005	0.01	0.08	0.24	0.34	2.27	0.04	6780
S28								0.0002	0.001							0.002		0.003	0.04	0.00	432000
S29																0.001		0.001	0.01	0.00	2456000
S30										0.0002	0.0002		0.00005			0.004	0.006	0.01	1.31	0.02	11800
S37										0.0001	0.0001					0.003	0.005	0.01	0.07	0.00	230000
S38			0.002		0.06	0.36	0.004					0.13				0.005		0.56	3.29	0.05	4690
S39																0.003		0.003	0.02	0.00	802000

R = retronecine base, H = heliotridine base

6

Chapter 6

General Discussion

6.1 Overview of results and main findings

The use of herbal supplements for sexual improvement and recreational purposes is rather a common practice in Ghana (Manortey et al., 2018). Many of these products are marketed through various outlets and sold in various forms (capsules, tablets, solutions) while others are presented in their crude state. Although the high accessibility, acceptability and assumed benefits of herbal products has promoted growth in the herbal industry and Ghana's economy at large (van Andel et al., 2012), the presence of adulterants and other contaminants of concern in these products and their potential adverse effects on the health of consumers cannot be underestimated.

In Ghana, herbal medicinal products are monitored and regulated by the Food and Drugs Authority (FDA-Ministry of Health) under the Food and Drugs Law, 1992. The objective of the FDA is to "provide and enforce standards for the sale of food, herbal medicinal products, cosmetics, drugs, medical devices and household chemical substances" [6]. The FDA considers herbal medicinal products as "any preparation containing raw plants or plant ingredients (with or without animal-derived ingredients) with acclaimed therapeutic or other human health benefit." The FDA is also responsible for the issuance of marketing authorization to producers who meet the required specifications with respect to product identity, proof of microbial, chemical and toxicological safety, the latter based on data on acute, sub-acute, sub-chronic and chronic toxicity. Besides these roles, the FDA is also responsible for marketing surveillance of products available to consumers.

All these measures show commitment on the part of the government in ensuring that the right rules and regulations are in place for their implementation by producers and manufacturers, in order to secure the safety of consumers. Nonetheless, in some instances, these established rules and regulations may simply be ignored by producers or poorly executed or simply inadequate to address all the issues related to sale and consumption of botanicals and their preparations available on the Ghanaian market. For instance, some producers may accidentally and/or unknowingly substitute or mix safe raw materials with potentially hazardous ones, while others may willfully adulterate less

[6] https://www.moh.gov.gh/foods-and-drug-authority/ accessed on 06/9/2021

effective plant products with active pharmaceutical ingredients at pharmacologically relevant levels in order to obtain the expected and claimed effects. Others also replace expensive raw materials with cheaper filler alternatives especially in the case of capsuled products, tablets and powders, thereby raising additional safety issues. Although some of these products do meet the legal requirements, others which do not, still end up in various sales outlets due to the actions of some unscrupulous producers.

This thesis is composed of six sections indicated by chapters. **Chapter 1** and **Chapter 6** present the introduction and discussion (respectively), while Chapters 2 through to 5 described the research performed to meet the 4 objectives of the study. The research was directed at the characterization of the potential hazards and risks of forty selected supplements collected from the Ghanaian market purported as highly efficacious in enhancing sexual performance (i.e. boosting of libido, increase in penile size, prolonged sexual endurance time, and improved sexual stamina). Information on the packaging labels indicated that most of the supplements served multiple purposes, but the most important questions to ask included 'Are they of natural constituents or do they contain non-natural active pharmaceutical ingredients (APIs)? Are the raw materials safe, or are they contaminated with plant constituents or process contaminant likely to have (long-term) adverse effects on the health of consumers?' All these questions needed scientific data to provide the right answers.

Based on these questions, the first objective of the thesis (**Chapter 2**) was to investigate the inhibition potentials of the selected supplements against phosphodiesterase type-5 (PDE-5) enzyme activity using the PDE-Glo bioassay. This is because high amounts of PDE-5 inhibition by active constituents in plants may potentially promote increased relaxation in smooth muscle cells and may lead to hypotension. Also, other studies have reported the high adulteration rate of supplements sold for sexual enhancement with active pharmaceutical ingredients (APIs) (i.e. synthetic phosphodiesterase type-5 inhibitors (PDE-5i)) by producers in order to meet the expected outcomes. This information guided the objective for **Chapter 3,** which was to determine the potential presence of APIs in the selected supplements using chemical analytical methods following the bioassay analysis in Chapter

2 together making up a tiered approach. **Chapter 4** and **Chapter 5** focused on contaminants present in the supplements and their associated health risks. In **Chapter 4**, the potential presence of polycyclic aromatic hydrocarbons (PAHs), polychlorinated dibenzodioxins/furans (PCDD/Fs) and dioxin-like polychlorinated biphenyls (dl-PCBs) were investigated using the DR CALUX bioassay and GC-HRMS analysis (i.e. a tiered approach). **Chapter 5** focused on the potential presence of pyrrolizidine alkaloids (PAs) by LC-MS/MS analysis. The risks related to consumer exposure to these chemicals were assessed based on the estimated daily intakes (EDIs) that were calculated to result from the use of the supplements taking into account the prescribed use and the levels of the ingredients of concern as quantified by the chemical analysis.

Upon screening the selected supplements for their PDE5 inhibition potentials using the PDE-Glo bioassay (**Chapter 2),** it was realized that, 36 (90%) out of the 40 supplements were able to inhibit PDE-5 enzyme activity at varying degrees. Estimated concentrations of active constituents expressed in mg sildenafil equivalents ranged from traces to more than100 mg/g. Eleven out of the 36 positive supplements were categorized as low (L; < 25 mg sildenafil equivalents), 16 as medium (M; 25 – 100 mg sildenafil equivalents) and 9 as high daily intake (H; > 100 mg sildenafil equivalents). Twenty-five supplements (62.5%) thus pointed at a daily intake higher than 25 mg sildenafil equivalents, while the recommended use of 9 (22.5%) out of these 25 supplements was found to result in EDIs higher than the maximum recommended daily intake of 100 mg sildenafil equivalents (based on the recommended daily dose of Viagra).

The responses observed in the bioassay for the 25 supplements categorized as medium (M; 25 – 100 mg) and high intake (H; > 100 mg) were unlikely to result from inherent plant constituents, and more likely caused by adulterants that were added on purpose by producers to obtain the expected outcome. This hypothesis is in line with reports from various studies which showed that natural products sold for sexual enhancement were regularly adulterated with APIs (Gratz et al., 2004; Zou et al., 2008; Wollein et al., 2011; Vaysse et al., 2012; Petal et al., 2014). Furthermore, there is hardly any scientific record of herbal products proven to contain high amounts of natural PDE-5i. In order to confirm the

hypothesis, the supplements were further investigated using LC-MS/MS analysis to detect and ultimately identify and quantify the presence of potential adulterants. The results of this study were presented in **Chapter 3**.

These further investigations revealed that sildenafil (main compound in Viagra) was present in the 9 supplements which exhibited high responses in the bioassay, while low amounts of tadalafil and vardenafil were also found in some samples. Generally, 13 out of the 40 supplements contained adulterants at levels above 1 mg sildenafil equivalents per gram of sample. When comparing the estimated concentrations in the bioassay to those determined by the LC-MS/MS analysis, it appeared that, for 35 out of the 40 supplements the concentrations obtained by the two methods were to some extent comparable (**Chapter 3**). This showed the accuracy of the bioassay in predicting negative or suspect (positive) supplements. However, clear discrepancies were observed for five samples of which one (S13) was further investigated to identify and characterize the PDE-5 inhibitor(s) present. For this, a preparative UHPLC analysis was performed by fractionating eluting chromatograms and further analysis of active fractions in the PDE-Glo bioassay. Subsequent LC-MS/MS and ^{1}H-NMR analysis revealed the presence of hydroxythiohomosildenafil, an analogue of sildenafil not yet included in the LC-MS reference library and therefore not detected in the first instance. Upon confirmation, the potency of the compound in the PDE-Glo bioassay was examined in comparison to sildenafil. The results revealed hydroxythiohomosildenafil to be substantially more potent than sildenafil with an IC_{50} of 80 nM as compared to the IC_{50} of 900 nM for sildenafil. This implied that hydroxythiohomosildenafil is about 12-fold more potent than its parent compound (i.e. sildenafil), thereby defining a relative potency (REP) value for hydroxythiohomosildenafil. The reference library of the HR-FS-LC-(Orbitrap) MS was subsequently updated to include hydroxythiohomosildenafil while the MS method was also optimized to quantitatively detect this additional PDE-5i. Next, the amount of PDE-5i in the sample (S13) extract was estimated in sildenafil equivalents considering the REP of hydroxythiohomosildenafil. The estimated concentration thus obtained by LC-MS/MS analysis was 12 mg sildenafil equivalents per gram of supplement, which was in line with the level of 10 mg

sildenafil equivalents per gram of sample estimated based on the effect of the 1000-fold dilution of sample S13 in the bioassay.

The purpose of these investigations was not to identify the responsible compounds in all five supplements with discrepancies between the bioassay results and the LC-MS/MS outcomes. It was rather to demonstrate as a proof-of-concept the use of a tiered approach in identifying (unknown) compounds (PDE5-i) present in herbal supplements, starting with screening of samples using the PDE-Glo bioassay, with a follow-up analyses of positive samples using chemical analytical methods. This showed the added value of using an effect based (bio)assay for the screening of samples for the presence of known and unknown active compounds. Results also illustrated the accuracy of the bioassay in presenting a reliable lead on what to expect and/or which samples needed further investigations using chemical analysis. In the end, the majority of the supplements exhibiting high inhibition potentials according to the PDE-Glo bioassay, were found to be adulterated with synthetic PDE-5i, with 12 supplements according to the LC-MS results containing concentrations resulting in intakes within and above pharmacologically relevant levels.

The evidences gathered in this study combined with literature data revealed the regular adulteration of herbal supplements sold as sexual enhancers. Although certain plants (e.g. horny goat weed, Thai ginseng, panax ginseng, rhodiola rosea, tribulus terristis and ginkgo biloba) are speculated to contain plant constituents able to enhance sexual performance, there is barely any scientific evidence to support this (Singh et al., 2012; Chaiyakunapruk et al., 2016; Ganapathy et al., 2020). The majority of the herbal products appeared to be adulterated with synthetic PDE-5i and/or their unapproved analogues to evoke the claimed effects (Koh et al., 2009; Singh et al., 2010; Mans et al., 2013; Reeuwijk et al., 2014; Gillard et al., 2015; Buckle et al., 2017; Popescu et al., 2017).

Amongst the adulterants, sildenafil and its analogues (e.g. homosildenafil (Shin et al. 2003); hydroxyhomosildenafil (Blok-Tip et al. 2004); acetildenafil (Blok-Tip et al. 2004, Shin et al. 2004); hydroxyacetildenafil (Hou et al. 2006, Park et al. 2007); piperidino acetildenafil (Gratz et al. 2006) and thiohomosildenafil (Venhuis et al. 2008, Zou et al. 2008)) are the most detected (Low et al., 2009;

Reeuwijk *et. al.,* 2013; FDA, 2017; Kumar et al., 2018). In the USA alone, meta-analysis by the FDA from 2007 to 2016 indicated that, out of 776 dietary supplements investigated, 353 marketed for sexual enhancement were adulterated; and 166 out of the 353 were specifically adulterated with sildenafil (Kumar et al., 2018). This is probably as a result of the readily described manufacturing process which may thus facilitate the illegal production resulting in a high prevalence of sildenafil and its analogues in herbal aphrodisiacs (Bell, Brown, & Terrett, 1990). Furthermore, sildenafil is the most conveniently available reference compound used by most food safety regulators and researchers, therefore its presence is probably also more easily detected compared to other less known analogues (Reeuwijk et al., 2013; Bujang et al., 2017). It was therefore not surprising to identify sildenafil as the major adulterant in most of the supplements.

The compound identified in S13 (i.e. hydroxythiohomosildenafil) is an analogue of sildenafil. Hydroxythiohomosildenafil was first isolated and identified, using ESI–MS/MS, NMR, UV and IR, in two herbal dietary supplements bought from the internet in a study reported by Koh et al. (2009). Although structurally similar to sildenafil, there are subtle structural differences such as a substitution of the oxygen atom with a sulfur atom in the pyrazolopyrimidine moiety, and a hydroxyethyl group attached to the piperazinyl nitrogen instead of a methyl group (Koh et al., 2009). Apparently, these subtle structural modifications substantially potentiate their PDE-5 inhibition at least in-vitro. Future molecular docking studies could possibly provide further insight in the molecular interactions of PDE-5 and inhibitor (i.e. sildenafil/hydroxythiohomosildenafil) at the active site of inhibition which may then explain the differences in potency.

The presence and high levels of sildenafil and its analogue (with even higher potency) do raise concerns, especially when already sildenafil is known to present various adverse health effects[7] and serious health risks to those who have suffered a stroke or recent heart attack, including individuals living with other serious heart or liver problems (Kostis et al., 2005). Use of sildenafil is also contraindicated

[7] https://www.drugs.com/sfx/sildenafil-side-effects.html accessed on 9/28/2021

in individuals taking organic nitrate drugs (e.g. nitro-glycerine) to treat angina, including any drug meant to lower blood pressure (Langtry & Markham, 1999; Ishikura et al., 2000). The combined intake of PDE-5i and organic nitrate drugs, α-adrenergic blockers and CYP3A4 inhibitors may synergistically promote increased relaxation of smooth muscle cells, resulting in a drastic reduction in systemic blood pressure (Kostis et al., 2005; Kloner, 2007). This may potentially lead to other cardiovascular effects and in extreme cases, sudden shock or even death (Langtry & Markham, 1999; Gur *et al. 2013*). In Ghana, many users of herbal supplements sold for sexual enhancement have met their untimely deaths, although due to lack of proper records, the numbers remain unknown even when reported cases continue to increase [8,9,10,11,12]. These products are mostly found in the possession of the deceased upon discovery and further investigations, providing the basis for the speculated link.

In totality, it is obvious that herbal products marketed for sexual enhancement are often adulterated with APIs with some at levels that may potentially lead to serious health risks especially for certain consumers with underlying conditions. Given the levels of the compounds detected, it is clearly not an issue of accidental cross-contamination, but rather one of deliberate adulteration. These observations justify the concerns raised by relevant stakeholders (i.e. medical health professional, regulatory authorities, consumers) and the call for necessary interventions.

Besides adulteration, other issues pertaining to the safety of botanicals, are the presence of environmental contaminants and also natural toxins. Contamination of raw materials with pollutants like PAHs, dioxins and dl-PCBs may result from use of raw materials contaminated during

[8] https://www.graphic.com.gh/lifestyle/life/man-hospitalised-with-3-day-erection-after-taking-viagra-meant-for-bulls.html accessed on 5/09/2021

[9] https://dailytrust.com/man-dies-in-hotel-few-minutes-after-using-sex-enhancers accessed on 5/09/2021

[10] https://www.ghanaweb.com/GhanaHomePage/africa/Man-dies-from-sex-pills-overdose-after-engaging-services-of-prostitute-861031 accessed on 5/09/2021

[11] https://dailypost.ng/2020/02/24/man-dies-after-taking-sex-enhancement-drugs-to-satisfy-lady-in-onitsha-hotel/ accessed on 5/09/2021

[12] https://www.pulse.com.gh/filla/man-66-found-lifeless-in-hotel-room-with-aphrodisiacs-as-lover-25-vanishes/ez2pwy1 accessed on 5/09/2021

planting/harvesting in polluted areas (O'Conner et al., 1990; Wild et al., 1992; Webber et al., 1994; Harvey 1997; Howsam & Jones 1998; Loutfy et al., 2010). Additionally, these contaminants may also be introduced into finished products as a result of certain processing methods (i.e. drying over open fires, smoking), or use of improper materials which favorably support the formation of these compounds.

In **Chapter 4**, the potential presence of PAHs, PCDD/Fs and dl-PCBs in the collected supplements were investigated. This was achieved by studying the response of engineered cells (rat hepatoma cell line (H4IIE-luc)) when exposed to less stable (e.g. PAHs) and more stable (e.g. PCDD/Fs, dl-PCBs) aryl hydrocarbon receptor (AhR)-agonists in sample extracts under conditions enabling both their detection. This screening was carried out using the so-called DR CALUX® bioassay while a confirmation of the presence and identification of the respective contaminants in ten supplements was carried out using GC-HRMS analysis. The combination of an acid-silica clean-up and long exposure duration (48 hrs) in the DR CALUX® bioassay, enabled the detection of the more stable AhR-agonists, whereas the less stable AhR-agonists were detected without an acid-silica clean-up and exposure of cells for only 4 hrs.

Cell responses at 4 hrs exposure to extracts prepared without an acid-silica clean-up were higher than those obtained from extracts with an acid-silica clean-up. At 48 hrs, cell responses for some extracts treated without an acid-silica clean-up were even higher than the response of extracts treated with an acid-silica column (with responses being close to the background). This indicated that the 40 supplements contained only low levels of stable AhR-agonists (e.g. PCDD/Fs and dl-PCBs), and relatively high levels of less stable AhR-agonists.

Based on these results, ten supplements (i.e. S1, S10, S14, S34, S36, and S40 (which showed high responses at 4 and 48 hrs exposure without acid silica), two supplements, S7 and S25 (which resulted in equal responses at 4 hrs with or without acid-silica), and two other supplements (S22 and S29) (with low responses at 48 hrs without acid silica) were selected for further characterization using GC-HRMS, for both confirmation and evaluation of the outcomes of the bioassay. Amongst the list of 24

PAHs in the GC-HRMS reference library, phenanthrene was abundantly identified in all ten samples with concentrations ranging from 2.98 to 94.30 ng/g. Fluoranthene and pyrene also contributed significantly to the sum of 24 PAHs. The 4 EU marker PAHs (i.e. benzo[*a*]anthracene, chrysene, benzo[*b*]fluoranthene and benzo[*a*]pyrene) proposed by the WHO (2005) and EFSA (2008) for risk assessments of PAHs ranged from not detected (ND) to 25.5 ng/g, where S29 recorded the highest Σ4PAHs (25.5 ng/g).

To further evaluate the bioassay responses, the GC-HRMS measured Σ4PAH concentrations were converted to their respective RLUs using the respective BaP dose-response curves at 4 or 48 hrs. The concentration measured in S29 (i.e. 25.5 ng/g) was expected to result in 139 RLUs at 4 hrs (4-fold above the background response of 38 RLUs), and in 32 RLUs at 48 hrs, (2-fold above the background response of 17 RLUs). These predicted RLUs based on the determined GC-HRMS concentrations detected for the Σ4PAH levels in the six supplement extracts could explain a substantial part of the DR CALUX® activity after 4 hrs but not at 48 hrs. The differences between the predicted responses (RLUs) based on the GC-HRMS and the determined responses according to the DR CALUX® bioassay, especially at 48 hrs, may be explained by the activity of other less stable (natural) AhR-agonists (Jeuken et al., 2003; van Ede et al., 2008). It is unclear if these could be a concern but in general the less stable natural AhR agonists are rapidly metabolized in vivo and do not accumulate in the body.

To estimate the daily intakes (EDIs) that would result from the use of the supplements, the quantified Σ4PAH levels were multiplied by the average weight of the recommended dose and by the number of doses per day, resulting in EDIs ranging from 7.2 to 111.3 ng Σ4PAH /day. Dividing the EDI by the average weight of a consumer (assuming an average weight of 70 kilogram) the estimated intake levels ranged from 0.10 to 1.59 ng Σ4PAH /kg bw/day (Table 2). Due to the genotoxic and carcinogenic nature of PAHs, the margin of exposure (MOE) approach established by EFSA (2008), was used to assess the potential health risks upon exposure to these contaminants and daily long-term use of the respective herbal supplements. This was achieved by dividing the $BMDL_{10}$ (i.e. 340 µg/kg

bw/day) of the lower 95% confidence interval of the dose that causes a 10% increase in tumor incidence established by EFSA (2008) based on a carcinogenicity study on coal tar mixtures by Culp et al. (1998) by the EDI. The MOE is the ratio between this $BMDL_{10}$ and the EDI. For compounds with a genotoxic and carcinogenic mode of action, the EFSA considers an MOE value larger than 10 000 as a low health concern whereas those close to or less than 10 000 are indicative of a potential concern for consumers' health and considered priority for risk management actions (EFSA 2005, 2008). The calculated MOE values for the supplements based on the EDIs were all above 10 000 (Table 2), thus indicating a low concern for consumer health.

Individual congeners of PCDD/Fs and dl-PCBs were generally below the LOQ. Mono-ortho and non-dioxin-like PCBs were not detected in any of the samples. However, there were detectable levels of 1,2,3,4,6,7,8-heptachlorodibenfuran (1,2,3,4,6,7,8-HpCDF), 1,2,3,4,6,7,8-heptachlorodibenzo-p-dioxin (1,2,3,4,6,7,8-HpCDD), octachlorodibenzodioxin (OCDD) and the non-ortho-PCB 77 in 6, 10, 9 and 10 supplements respectively. Nonetheless, these congeners are noted for their low toxic potencies, as reflected by their TEF values (Van den Berg et al., 2006).

The quantified GC-HRMS data were expressed in toxic equivalents by multiplying the concentration of each congener by its respective toxic equivalency factor (TEF) and summing up to determine the total concentrations in pg TEQ/g of supplement (Van den Berg et al., 2006). Accordingly, the concentrations of PCDD/Fs and dl-PCBs in the samples (based on the lower bound) ranged from 0.01 to 0.19 pg TEQ/g. According to Loutfy et al. (2010), the differences in PCDD/Fs and dl-PCB concentrations (pg WHO TEQ/g) in medicinal plants, is likely due to the burden of congeners in different plant locations and differences in the accumulation capacities of the plants. In practice also, certain processing methods, involving the exposure of products to high temperatures and low oxygen may predispose finished products to different amounts of these unwanted compounds. The low amounts of PCDD/Fs and dl-PCBs corresponded with the observed low responses in the DR CALUX® at 48 hrs exposure to extracts treated with an acid silica clean-up. The ones that did show

a low response at 48 hrs with acid-silica, i.e. S10, S14, S29 S36 and S40 were samples that according to the analytical data, contained some PCDD/Fs plus dl-PCBs.

The estimated daily intakes (EDIs) (pg TEQ/day) based on the total PCDD/F and dl-PCB concentrations (pg TEQ/g) in the supplements ranged from 0.01 to 1.20 pg TEQ/day. This resulted in exposure levels ranging from 0.0001 to 0.02 pg TEQ/kg bw/day. Assuming daily use, the calculated daily exposures translates to a weekly intake of 0.001 to 0.13 pg TEQ/kg bw/week, and are thus substantially lower than the established TWI of 2 pg TEQ/kg bw/week (EFSA, 2018), indicating a low concern for consumers' health.

Another group of contaminants of concern often present in botanical supplements are a group of secondary plant metabolites considered as an important category of natural toxins, the pyrrolizidine alkaloids (PAs). PAs are present in over 6000 plant species and more than 3% of flowering plants (Smith et al., 1981). PAs are known genotoxic carcinogens which may be naturally present in plants intentionally used as raw materials for supplements. They may also be introduced unintentionally when PA containing weeds are co-harvested with the raw materials, or due to growing conditions resulting in uptake from neighboring plants via soil (Selmar et al., 2019; Hama J.R. and Strobel B.W., 2021).

In the **Chapter 5** of this thesis, the presence of plant toxins belonging to the group PAs in the series of 40 supplements were identified and quantified using LC-MS/MS analysis. All 40 supplements were analyzed for the presence of sixty-four 1,2-unsaturated PAs. The focus was on 1,2-unsaturated PAs because these PAs are especially known to be both genotoxic and carcinogenic (Pereira et al., 2018; Hessel-Pras et al., 2019; Yang et al., 2020).

The results obtained revealed the presence of seventeen different PAs in 19 out of the 40 supplements. In decreasing order, the most detected PAs were rinderine (in 15 samples), its *N*-oxide (in 10 samples), intermedine (in 11 samples), its *N*-oxide (in 11 samples), lycopsamine (in 8 samples) and its *N*-oxide (in 5 samples). The rest of the PAs appeared in less than 5 supplements. This suggests the contamination of some selected supplements with specific weeds rather than the use of PA-containing

herbs, which is also with regards to the observed levels. The concentrations of total PAs ranged from 0.005 to 3.2 µg/g, which resulted in exposures from 0.01 to 12 µg/day, corresponding to 0.0002 to 0.2 µg/kg bw/day (assuming consumption by a 70 kg person). When compared to a $BMDL_{10}$ of 237 µg/kg bw/day for the induction of liver tumours in rats treated with riddelliine (EFSA 2017), the MOE values ranged from 1 200 to 1 400 000 where the use of 8 supplements resulted in MOE values below 10,000, thus implying a health concern upon daily use for a prolonged period of time.

Similar investigations have been reported for other medicinal botanical preparations (Bah et al., 1994; Roeder and Wiedenfeld, 1996; 2009; 2011; 2013; Chen et al., 2017; Kaltner et al., 2020; Suparmi et al., 2021). In a study by Bodi et al. (2014) of PAs with concentrations ranging from < LOD to 5647 µg/kg were detected in tea, herbal drugs and honey using LC-MS/MS. They concluded that the presence of PAs in samples was most likely the result of contamination when raw materials are co-harvested with PA-producing plants. Roeder (2000) reviewed the presence and toxicity of various PAs in 38 plants used in traditional Chinese medicines belonging to the plant families *Orchidaceae, Fabaceae*, *Boraginaceae* and *Asteraceae* while evaluating their metabolism and toxicity. Also, in a study by Wang et al. (2021) 271 out of the 386 different Chinese herbal medicines listed in the Chinese Pharmacopoeia (2020) were found to contain PAs with concentrations ranging from 0.1 to 25 567 µg/kg. They concluded that 10 out of 271 positive samples would result in MOE values below 10 000, thus indicating priority for risk management actions. The evidences gathered in the **Chapter 5** combined with literature, show the prevalence of PAs in medicinal botanical preparations. Although some supplements were produced with PA-containing plants, unintended cross-contamination seemed the major source of these plant toxins. The results obtained provide convincing data to support risk management actions in Ghana on the presence of PAs in botanicals and botanical preparations in order to better guarantee the safety of herbal products sold for improving sexual performance.

6.2 General discussion

In this thesis, the inhibition potentials of herbal supplements sold for improving sexual performance were investigated using the PDE-Glo bioassay, while confirmation and quantification was carried out

by chemical analysis. Although the responses from the bioassay pointed at the inhibition potentials of 36 out of the 40 supplements at varying degrees, the LC-MS/MS analysis revealed the presence of added APIs in some supplements, with estimated daily intakes within and even above pharmacologically relevant levels. The adulterants present in these supplements were not the only contaminants of concern, but also the prevalence of PAs, PAHs, PCDD/Fs and dl-PCBs. The levels of PAHs, PCDD/Fs and dl-PCBs were generally low and were not a concern for risk management actions, however the levels of PAs in 8 supplements indicated a priority for risk management actions.

Although the procedures employed in this thesis (i.e. sample selection, bioassays, analytical methods, and risk assessment) did successfully provide answers to the various objectives, the employed procedures also presented some challenges. In the following section, the challenges encountered during analysis will be addressed from a broader perspective while presenting some solutions for future consideration and subsequent improvement.

These include:

- Selection of samples based on a targeted sampling approach.
- Extraction of bioactives present in supplements.
- Analytical methods (i.e. biological assays and chemical analysis) employed for the detection, confirmation and quantification of bioactives.
- Risk assessment procedures and alternatives for genotoxic carcinogens.

6.2.1 Selection of samples based on a targeted sampling approach

In the present thesis the main criteria for sample selection was related to herbal products marketed for improving sexual performance. Hence, selection of samples based on their composition for the presence of PAs, or processing methods used with regards to PAHs, PCDD/Fs and dl-PCBs, were not considered during sample selection. Sample selection based on a targeted sampling approach, by collecting supplements for which the processing method was by drying over open fires, or samples containing herbal constituents from a list of PA-containing plants as indicated on the packaging labels

would have increased the likelihood of detecting these contaminants of concern. Thus, targeted sampling provides a preferred way to detect the presence of potentially high levels of constituents of concern as compared to the general assumption that these contaminants (PAHs, PAs) could be detected in the supplements even when collected by a non-targeted sampling approach. This may explain why the levels of these constituents in the 40 samples analyzed in the present thesis were relatively low when compared to the levels in other studies (Wang et al., 2021). As a downside, targeted sampling may exaggerate the occurrence of such contaminations in plant products. Nonetheless, the results obtained in this thesis serves as an indication of the level of contamination in herbal products, while at the same time acknowledging that these contaminants will likely be detected even at higher levels and more frequently when sampling would be carried out based on a targeted approach for these contaminants of concern.

6.2.2 Extraction of bioactives in supplements.

The most crucial step in the successful detection of unknown compounds and/or bioactives in complex mixtures is the selection of the right solvents for extraction. The criteria for solvent selection include the distribution coefficient, selectivity, recoverability, and chemical reactivity amongst many others[13]. In **Chapter 2**, extracts were prepared with a mixture of acetonitrile/water (ACN/H_2O (80/20, v/v)), which is one of the commonly used solvents for the extraction of bioactive compounds like sildenafil and its analogues (Amaral et al., 2015). However, the choice of the solvent for extraction is always a challenge when aiming at the detection of unknown compounds or bioactives. For instance, due to the different solubility profiles of the different PDE-5i commonly used in adulterating botanical preparations, the application of ACN/H_2O may potentially limit the general dissolution of all compounds of interest, thus preventing their detection. It is also reported that certain groups of PDE-5i (e.g. tadalafil and avanafil) are practically insoluble in water and only slightly soluble in

[13] http://www.separationprocesses.com/Extraction/SE_Solvent.htm accessed on 12/08/2021

organic solvents (Amaral et al., 2015). As a result, part of these compounds (if present in the supplements) may be poorly dissolved in ACN/H_2O, thereby affecting the outcomes of both the bioassay and the LC-MS/MS analysis and hence the conclusions drawn. The same extracts used for screening in **Chapter 2** (PDE-5i screening) were also used for further studies in **Chapter 3** (LC-MS/MS analysis) in order to ensure consistency between the two results. In future studies, one might consider the use of a broader range of solvents for extraction, especially when looking for unknown compounds of concern. Preferably, the selection of a mixture of more polar solvents (e.g. methanol/water/acetonitrile) or the use of two extraction solvents, i.e. preparing both a rather polar and an a-polar extract of one sample and analysis of both extracts while finally selecting the best option, might turn out to be of use. While optimizing the extraction solvent, it should also be kept in mind that the percentage of solvents in a mixture other than water should not be at levels affecting the applied bioassays.

6.2.3 Analytical methods (i.e. bioassay and chemical) employed for the detection, confirmation and quantification of bioactives.

Prior to the advocacy of alternative methods, animal studies were mainly used in determining the efficacy of plant products during drug development. This mostly involved the exposure of animals to test compounds vis-a-vis controls, which was usually cost intensive in addition to being considered unethical. As a result, various bioassays were developed as alternatives to testing bioactive components in complex mixtures including botanical preparations to determine their beneficial (e.g. antioxidant properties) and adverse (e.g. genotoxicity) effects (Prinsloo et al., 2017). A review by Prinsloo et al., (2017) discussed in detail various bioassays, their advantages (i.e. rapid, less expensive) and disadvantages (i.e. possibility of false-positive and false-negative outcomes, requirement of extensive sample clean-up and easy interferences by other active ingredients). However, chemical analytical methods suffer from the same issues as well. Another advantage of bioassays is its ability to detect yet unknown bioactive compounds (**Chapter 2**) and that it gives a total activity of a complex mixture (**Chapters 2, 3 and 4**), however, the latter is a drawback, as bioassays cannot identify the specific compounds eliciting the response. Bioassay outcomes thus

often need additional confirmation, identification and quantification of suspect compound(s) by chemical analytical methods.

Nonetheless, there has been instances where chemical analytical methods have been less efficient in detecting compounds in complex mixtures as found in crude botanical extracts. In such instances, bioassays appear more useful and suitable in the identification of inherent plant properties in complex mixtures. Although the level and nature of individual compounds detected by chemical analysis may vary with the batches analyzed, the bioactivity status often remains the same. Thus, chemical analysis of a single component in mixtures may not be very informative (McLaughlin et al., 1998) especially when their biological effects remains unknown. Furthermore, the quality of feedback from chemical analysis strongly depends on the nature of compounds to be analyzed, the solvent used in the mobile phase, the flow rate and the type of interface employed (Petal et a., 2010). However, due to the different physicochemical properties of crude natural products, including the various solubilities, molecular sizes and stability, it is often a challenge and in some cases almost impossible to optimize the ionization conditions to suit all the different known/unknown compounds. This, according to Wolfender et al. (1995) can be solved by analyzing extracts in different ionization modes, which makes it extra expensive. The application of a tiered approach where a bioassay is first used to screen samples to assess the total effect in complex mixtures, followed by chemical analysis for confirmation and quantification of the bioactive compounds is a preferred option. This enables the detection of potentially unknown compounds, as demonstrated in **Chapter 3**.

In **Chapter 2,** the PDE-Glo bioassay was used to screen the samples to determine their inhibition against PDE-5 enzyme activity. A similar bioassay approach was employed in a study by Santillo and Mapa (2018). One major advantage of this bioassay was its ability to screen all the supplements in one runtime (i.e. 120 minutes) (high-throughput). Additionally, the bioassay is not complicated and rather easy from start to finish. At the end, luminescence is measured directly after 20 minutes of adding the last reagent (kinase-glo). However, one common challenge encountered when using bioassays are interferences by sample matrices, which was also the case in the study described in

Chapter 2. It was observed that undiluted extracts of solid samples showed high erroneous responses and thus potential false-negative results (Fig. 1). Nevertheless, upon diluting these samples, matrix interferences were reduced, thereby allowing analytes of interest to properly interact with reagents, thus resolving the false-negative outcomes to a large extent. It was actually shown that due to the shape of the dose-response curve, sample extracts required at least a 100-fold dilution to allow the selection of samples that could contain levels resulting in doses above the therapeutic dose of 25 mg/day. Luckily the PDE-5 enzyme assay is very sensitive and inclusion of 1,000- and 10,000-fold dilutions still allowed a good estimate of the level, as shown by the comparison with the LC-MS/MS determined levels.

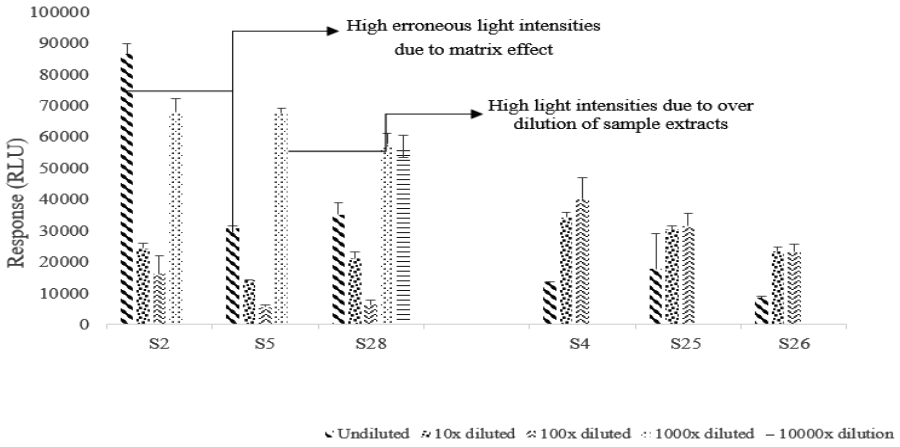

Fig. 1: Effect of dilution of sample extracts (3 solid (S2, 5 and 28) and 3 liquid (S4, 25 and 26)) and their corresponding responses (mean ±SD) in the bioassay

The strength of the bioassay is in its ability to detect the general activity of both known and unknown active compounds contrary to chemical analysis which only detects compounds that are available in a reference library (Stranska-Zachariasova et al., 2019). However, the PDE-Glo bioassay being a screening tool, only determines the biological response/activity of enzymes (qualitatively or semi quantitatively) upon exposure to test compounds and lacks the ability to identify the compound(s) eliciting the observed responses. Application of bioassays therefore generally requires a follow-up

consisting of a chemical analysis of "suspected" samples for the identification of the responsible compounds causing the observed response and further confirmation and quantification. The goal of many chemical analytical techniques has been to identify and quantify known compounds of interest in botanical samples. Nevertheless, these chemical analytical methods also present their own challenges including expensive nature of columns and solvents used, the lack of long term reproducibility due to column packing, their inefficiencies in the analysis of complex mixtures, the need for highly skilled personnel, often longer total run-time for series of samples, and the reliance on a wide range of reference standards which sometimes may not be readily available (Patel et al., 2010; Vaclavik et al., 2014). In addition, some compounds are difficult to measure by mass spectrometric methods and these methods often suffer from matrix effects too. That notwithstanding, chemical analytical procedures have been widely applied in the identification and quantification of PDE-5i in herbal supplements sold for improving sexual performance (Gratz et al., 2004; Zou et al., 2008; Wollein et al., 2011; Vaysse et al., 2012; Petal et al., 2014).

It is obvious that the identification of compounds based on chemical analytical methods may be limited by the availability of compounds in the reference library. Moreover, due to the on-going production of new analogues for adulteration purposes, existing reference libraries may not be up-to-date with respect to newly developed compounds. Hence, most of the reported studies based on chemical analytical analysis only may still include false-negative reports as demonstrated in **Chapter 3**. It was realized that without the bioassay results, S13 would have easily passed as a negative sample (i.e. containing no adulterant) which would have been a false-negative report. In some instances, existing spectral data of known compounds in literature may be used to confirm the structure of the identified mass of an unknown compound. However new analogues may be identified by a series of analytical methods as demonstrated in **Chapter 3**. Elucidation of compound structures after isolation using NMR and mass spectroscopy has also been applied in previous studies (Shin et al., 2003; Lee et al., 2011; Kee et al., 2012; Schramek et al., 2014; Malet-Martino et al., 2015; Fu et al., 2020). Mustazza and others (2014) synthesized and characterized sildenafil and thirteen of its analogues

using NMR and MS/MS spectra. In another study by Bong-Jin et al. (2011), the structure of a vardenafil analogue (acetyl vardenafil) present in megaton (a dietary supplement) was detected by high-performance liquid chromatography (HPLC) analysis with a photodiode array detector (PDA), and elucidated by mass spectrometry (MS), infrared (IR) spectroscopy and one- and two-dimensional nuclear magnetic resonance (NMR) spectroscopy.

A tiered approach, involving the use of a bioassay followed by chemical analysis of "suspected" samples, serves as a proof-of-concept in solving the limitations encountered when using chemical analysis in isolation, while providing at the same time more scientific evidence on the safety of these herbal products based on the bioassay response. This is aimed at increasing consumer confidence, thus paving the way for incorporation of botanicals into legitimate, long-term health practices (Akshay et al., 2013).

In **Chapter 4**, the DR CALUX® assay was used for initial screening of the supplements. Like any other assay, it provided the benefits of screening large numbers of samples (high-throughput) in a relatively shorter time, with high sensitivity to compounds activating the aryl hydrocarbon receptor (AhR)-mediated signaling pathway. Due to its sensitivity to all compounds that can activate the AhR, the assay may be potentially hindered by the lack of specificity. The high sensitivity and lack of specificity of the DR-CALUX® assay has been addressed by Hoogenboom et al. (1999) and a review by Windal et al. (2005). In this review, the authors indicated that the widely applied acid silica clean-up during sample pre-treatment improves purity and limits interferences of especially naturally occurring compounds in food and feed including PAHs, while increasing the detection of stable compounds like dioxins and dl-PCBs. However, the extensive and laborious nature of the sample pre-treatment using acid silica clean-up is a challenge. Additionally, the acid-silica clean-up procedure requires the use of large amounts of solvents (hexane) and other reagents making it relatively expensive. That not-withstanding the DR CALUX® assay is robust, relatively cheaper when compared to chemical analysis, and fully validated for several food and feed matrices.

The PDE-Glo (**Chapter 2**) and the DR CALUX® (**Chapter 4**) bioassays were applied to test samples to assess their biological effects and to subsequently select samples/supplements that needed further analysis using hyphenated chemical analytical techniques for identification and quantification of responsible compounds. The PDE-Glo bioassay rightly predicted the positive samples with high PDE-5 inhibition potentials, while the chemical analysis identified the responsible compounds and their concentration levels in most of the samples (**Chapter 2 and Chapter 3**). Similarly, the DR CALUX® assay correctly predicted the low levels of stable (i.e. PCDD/Fs and dl-PCBs) and the probable presence of less stable (i.e. PAHs) AhR-agonists in the test samples, as confirmed by the GC-HRMS (**Chapter 4**). In both instances, the outcome of the bioassays served as a positive lead for samples that needed further investigations thus proving their reliability for screening samples and aiding the selection of those samples that needed confirmation and quantification of their bioactive constituents.

6.3 Risk assessment procedures employed in the various chapters.

The risk assessment approaches employed in the different chapters in this thesis followed different procedures. For compounds with a non-genotoxic mode of action i.e. PDE-5i (**Chapter 2** and **Chapter 3**) the risk assessment followed the comparison of the EDIs to recommended therapeutic doses of Viagra, whereas the EDIs of PCDD/Fs and dl-PCBs (**Chapter 4**) were compared to a health based guidance value (i.e. the tolerable weekly intake (TWI)) established by the European Food Safety Authority (EFSA) (2018). The decision to compare the EDIs of PDE-5i in the selected supplements to the recommended therapeutic doses of Viagra (with sildenafil as the active ingredient) was based on the fact that sildenafil was the most commonly detected adulterant in most sexual enhancing herbal products which was also the case for the samples analyzed in the present thesis. As a result, sildenafil was selected as the reference compound in the PDE-Glo bioassay **(Chapter 2)**. However, some PDE-5i (e.g. tadalafil, vardenafil) are noted to be more potent than sildenafil (Bischoff, E., 2004) and this appeared to be also the case for some analogues of sildenafil (e.g. hydroxythiohomosildenafil) as demonstrated in **Chapter 3**.

Accordingly, the estimated compound concentrations in the supplements were expressed in mg sildenafil equivalents per gram of sample. This was used to estimate the daily dose, thus enabling the comparison to the therapeutic doses of sildenafil to evaluate the potential health effects of the supplements. To further allow a risk assessment, further details on dose levels causing adverse health effects in subgroups of sensitive individuals would be of use. At the current state-of-the-art, a daily intake of 100 mg is considered a dose level that may result in adverse effects, especially in sensitive individuals. However, what would be a safe level for these sensitive subgroups is yet to be established. Given the general approach in risk assessment to use a factor of up to 10 to extrapolate a lowest observed effect level (LOAEL) to a no observed adverse effect level (NOAEL) (EFSA, 2012), one could argue that such safe levels would be up to 10-fold lower than the dose level of toxicological concern. The default uncertainty factor of 100 for inter- and intraspecies differences may not be required given that the LOAEL would be derived from the dose level causing adverse effects in sensitive human individuals. However, it should be stressed that the adverse effects are in some cases very severe including fatal outcomes.

Assuming that safe levels for the sensitive subgroups in the population would be 10 mg/day, one can conclude that based on the outcomes of the bioassay 25 (62.5%) out of the 40 supplements (**Chapter 2**), would result in intakes above this level and raise a concern for sensitive individuals. Based on the LC-MS outcomes, the estimated sildenafil equivalent intakes of 12 (30%) out of the 40 supplements (**Chapter 3**) would result in intakes above this level and raise a concern for sensitive individuals. The lower number obtained based on the LC-MS as compared to the bioassay data is partly due to the use of a cut-off value in the LC-MS analysis and the presence of unknown compounds responsible for the effect in the bioassay in some instances.

For PAHs, (**Chapter 4**) and PAs (**Chapter 5**) with a genotoxic and carcinogenic mode of action underlying their toxicity, their regulation was carried out with the assumption that they may increase cancer risk in consumers upon repeated daily exposure even at low levels. As a result, there is no established safe exposure thresholds like the tolerable daily intake (TDI) for PCDD/Fs and dl-PCBs.

The risk assessment of chemicals that are genotoxic and carcinogenic can be performed using the Margin of Exposure (MOE) approach proposed by EFSA (2005). The MOE is the ratio between the lower confidence limit of the benchmark dose causing 10% effect above background levels (i.e. $BMDL_{10}$), derived from mathematical modelling of the available data on the carcinogenic effects of a compound in experimental animal studies, and the estimated daily intake (EDI).

According to the EFSA Scientific Committee, an MOE of 10 000 or more, is considered of low concern from a public health point of view and might reasonably be considered as a low priority for risk management actions (EFSA, 2005). Such a conclusion was drawn in **Chapter 4** for the consumer exposure to the levels of PAHs in the supplements. A similar conclusion was drawn in **Chapter 5** for 11 out of the 19 positive supplements (out of the 40 analyzed) that tested positive for the presence of PAs. However, the PA levels in 8 supplements containing PAs would result in intakes that give rise to MOE values below 10 000 indicating a potential concern upon long-term exposure and a priority for risk management actions.

The MOE values calculated in **Chapter 5** for the combined PA exposure were based on the total sum of PAs, in which the level of all PAs was added up without considering potential differences in potency. However, a refined risk assessment procedure using the interim relative potency factors (iREPs) of the different PAs proposed by Merz and Schrenk (2016) was also performed. Upon this reanalysis, 2 supplements still were found to result in EDI values that would give rise to MOE values below 10 000, thus indicating a priority for risk management actions.

It is also important to note that the MOE approach as suggested by EFSA and applied in the present thesis assumes a chronic exposure when assessing the risk upon exposure to genotoxic and carcinogenic compounds. However, in real life scenarios, the use of these supplements for improved sexual performance may rather depend on as-and-when-needed and thus relate to a shorter and occasional exposure. Therefore, assessing the risk using the MOE approach may potentially overestimate the risks to these compounds when assuming chronic exposure. Thus, an alternative approach would be necessary. Although at present there is no widely accepted method to take such

infrequent exposure into account, in a study by Chen et al., (2017), the Haber's rule was applied to correct for shorter than lifetime exposure (Gaylor, 2000). The Haber's rule holds the assumption that the incidence and/or severity of a toxic effect is the same when the total exposure concentration (c) ,times the duration (t) of exposure (c x t) is similar (Crump et al., 1976). When this approach is applied in the risk characterization of PAs in **Chapter 5**, none of the 19 PA containing supplements will result in an MOE value below 10 000 (assuming an intake of once per week, resulting in 52 days a year by a regular consumer). Although Haber's rule can be applied as a first-tier approach to estimate the consequences of shorter-than-life-time exposure, it is at present not generally applied for risk assessment of genotoxic carcinogens by risk assessors. The presence of PAs in supplements like the ones studied in the present thesis illustrates however, that such a widely accepted approach for correcting for shorter than lifetime exposure when using the MOE approach is needed to avoid overestimation of the risks.

Furthermore, it is also of importance to note, that the MOE approach is based on the premise that values below 10 000 are considered a priority for risk management actions while MOE values above 10 000 are considered as low priority for risk management actions. Nevertheless, there are instances where MOE values fall below or above 10 000 by several orders of magnitude and yet are evaluated as exposures for which MOE values are just below or just above 10 000. For instance, in **Chapter 5** the MOE values upon exposure to PAs in supplements ranged from 1 241 to 1 418 092. Those below the MOE cut off value of 10 000 ranged from 1241 to 8475 whereas those above 10 000 varied from 10 567 to 1418092. The 1241 was 8-fold lower, while 8475 was only 1.2-fold below 10 000, yet they were assessed as though they both indicate a priority for risk management. Similarly, 10 567 was 1.1-fold above, whereas 1 418 092 was more than a 100-fold above 10 000. Given that the MOE is a tool to set priorities in risk assessment these differences can be used to define higher priorities for supplements with the lowest MOE values. This situation however also indicates the necessity to define a certain banding of the MOE values.

The Committee on Carcinogenicity of Chemicals in Food, Consumer Products and the Environment (COC) in 2012 proposed a banding system for risk assessment based on the MOE values. In this document, an MOE value below 10 000 is considered of 'concern', values from 10,000 to 1,000,000 are considered 'unlikely to be of concern' while values above 1 000 000 are considered 'very unlikely to be of concern'. Based on this banding, 8 of the 19 PA-containing supplements would be a concern, 10 would have been considered 'unlikely to be of concern' and 1 supplement would have been considered 'very unlikely to be of concern'. Although banding for MOE values above 10 000 were defined, MOE values below 10 000 were general considered 'of concern', nonetheless there were substantial differences. Thus, one could propose that MOE values below < 1 000 may be defined as 'very likely of concern', those ≥ 1 000 to 5 000 considered 'likely of concern', while those > 5 000 to 10 000 considered as 'of concern'. Following these proposed banding for Chapter 5 on PAs, none of the supplements would be considered 'very likely of concern', six supplements would be considered 'likely of concern', and two supplements would be considered 'of concern'.

6.4 Finally, the following section presents some **future perspectives** on how to expand the present studies to other herbal products available on the Ghanaian market, with the aim of building a strong database as the way forward towards the performance of risk assessment for the establishment of exposure limits for compounds of concern in consumer food products.

The future of risk assessment and risk management of herbal supplements in Ghana

In this thesis, aside investigating the safety of the selected supplements, another reason for performing the studies was to employ well-established, efficient, less expensive and less complicated experimental procedures that can be easily set up in laboratories and applied to other consumer products available on the Ghanaian market. This was to serve as a foundation for data gathering as a way forward in the establishment of intake limits and subsequent risk assessment of consumer products to enhance their safety.

In Ghana, the regulation of consumer food products including herbal supplements, is mainly under the supervision of the Food and Drugs Authority and Ghana Standards Authority. These two authorities established by the government, protect and promote public health through the control and monitoring of food products, medicinal drugs and medical devices, herbal and dietary supplements, animal foods and feed including veterinary products. Nevertheless, the safety guidelines and regulations by these authorities seems wholly inadequate, given that there are no established limits guiding these safety protocols for consumer products as available and implemented in for example the European Union (EU). In Europe, the European Commission defines the regulation of consumer products based on scientific data/evidences provided by the European Food Safety Authority (EFSA). The EFSA comprises of 15 independent management members and 10 scientific panels, and is responsible of the organization of expertise and the gathering scientific data to provide independent, up-to-date scientific advice on food safety issues, and communicating its scientific findings to the public. The EFSA cooperates with EU countries, international bodies, and other stakeholders, with the provision of independent scientific advice on existing and emerging food related risks, thus informing European laws, rules and policymaking with the ultimate aim of protecting consumers [14]. Currently, there is no such independent food safety authority in Ghana or close neighboring African countries, with the ability of providing useful data for risk assessment given the similarities in consumer products. In Ghana, the risk assessment of consumer exposure to unavoidable contaminants of concern as the ones identified in **Chapter 4** and **5,** are mostly carried out based on established limits, including health based guidance values and/or points of departure (POD) established by the WHO and or EFSA. Nevertheless, due to inter-racial differences, and differences induced by geographical locations and consumption patterns and habits, the dynamics and/or kinetics of these compounds of concern from one location to another may vary. As a result, assessing risks based on limits set by other countries for situations in Ghana may be flawed and/or require other uncertainty factors. The actual existence of potential differences in toxicokinetics and/or toxicodynamics between

[14] https://europa.eu/european-union/about-eu/agencies/efsa_en accessed on 8/10/2021

different ethnic groups was demonstrated in a study by Ning et al. (2017), in which the differences between Chinese and Caucasians in terms of metabolic bioactivation and detoxification of the food-borne genotoxic carcinogen estragole were characterized using physiologically based kinetic (PBK) modelling. Similarly, Zhao et al. (2019) reported differences between Chinese and Caucasians in relation to sensitivity towards the organophosphate pesticide chlorpyrifos, resulting in slower bioactivation of chlorpyrifos to chlorpyrifos-oxon and faster detoxification of chlorpyrifos-oxon in Chinese than Caucasians. It would be of interest to investigate whether similar differences in sensitivity exist between Caucasian and African populations. Additionally, the use of new approach methodologies (NAMs) including physiologically based kinetic (PBK) modelling will prove essential to providing answers to these questions, since they allow use of in vitro and in silico techniques to elucidate the interethnic variations (Chen et al., 2017; Zhao et al., 2019).

A PBK model comprises of a set of mathematical equations involving input parameters such as physiological parameters (i.e tissue volumes and blood flow), physico-chemical parameters (i.e. blood/tissue partition coefficients) and kinetic parameters (i.e. kinetic constant for metabolic reactions) (Rietjens et al., 2011; Li et al., 2017; Louisse et al., 2017; Zhang et al., 2018). These parameters aid in the description of chemical kinetics in the in vivo situation enabling studies of the overall effect of species, individual and/or ethnic differences in kinetics parameters for relevant metabolic conversions for the overall kinetic outcome and the resulting toxicity, thereby enabling prediction of chemical toxicity by an efficient new approach methodology for future risk assessment (Bessems et al., 2014).

Nonetheless, it should be noted that a PBK model-based approach cannot be directly applied to complex mixtures such as botanicals and botanical preparations. However, upon identification and quantification of compounds of interest (e.g. PAs), endogenous concentrations of these constituents resulting from estimated intake levels upon use of the botanical products can be predicted, to determine how much of this compound will be available in the blood and target tissue (i.e. the liver), enabling further evaluation of the risks following these internal dose levels in humans.

The evidences gathered based on literature, points at the advancement in food safety issues and the progress made by other developed countries towards the improvement of risk assessment methods and procedures. Nonetheless, Ghana and Africa seems to be lagging behind. There is a need to take baby steps by first gathering relevant scientific data, to establish a secured rich database ultimately aimed towards the establishment of an independent scientific body/committee responsible for setting health based guidance values and/or points of departure (POD) for Ghana and possibly other African countries for the performance of risks assessments and relevant exposure limits for compounds of concern in consumer products. This will go a long way in aiding the decision-making process which involves integrated considerations on political, economic and legal concerns for risk management actions, towards the reduction of risks to acceptable levels.

6.5 Conclusions

The present thesis provides insight in a number of relevant safety concerns for a series of 40 supplements sold for improving sexual performance on the Ghanaian market. The results obtained revealed that one cannot depend solely on chemical analysis for the detection of adulterants and contaminants in these herbal supplements. The use of a tiered approach, where a bioassay is first used to screen samples to assess their bioactive components while providing a positive leads on prioritize samples for further investigations, followed by chemical analysis for the identification and quantification of responsible compounds, is best suited for analysis of compounds in complex mixtures including botanicals and botanical preparations.

Based on the levels of constituents of concern and their estimated daily intakes, it appeared that there is a need for increased public awareness about adulteration, and the presence of harmful contaminants, and their potential consequences on the health of consumers of herbal supplements promoted for sexual enhancement. It is important to note that this study also indicates that the use of these supplements require caution.

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Summary

Summary

The reliance on herbal medicinal plants by consumers as an alternative to their primary healthcare needs is a common practice in Ghana. This is due to engrained socio-cultural practices, strong media persuasion, financial constraints amongst many others. Although most of these products are regulated to some extent by relevant authorities and sold legally, a good number of them are also marketed illegally by some unscrupulous producers. Consumers, being more focused on the benefits of these products, pay little to no attention to the potential health risks. These products, presented in various forms and marketed for various diseases and ailments, are also sold as supplements meant to compliment certain needs of consumers.

Amongst the popular ones are herbal supplements sold for improving sexual performance. These supplements are claimed to be highly efficacious in achieving the intended purpose, but there have been several instances where consumers are faced with various health challenges including for example priapism[15] and in worse cases even death upon use of these products, although there has been no direct medical proof of these deaths upon the use of such products. Usually victims are found dead with these products in their possession, hence the speculated connection of their death with the use of these products. There are no existing safety evidences regarding the use of these herbal products, nonetheless they are readily available in various sales outlets.

In the current thesis, the inhibition potentials of 40 herbal supplements sold in Accra (Ghana), for improving sexual performance were investigated using the PDE-Glo bioassay. Subsequently, the presence of synthetic PDE-5i added on purpose by producers to achieve the expected outcomes was determined using various chemical analytical procedures. Additionally, the presence of other

[15] https://www.graphic.com.gh/lifestyle/life/man-hospitalised-with-3-day-erection-after-taking-viagra-meant-for-bulls.html accessed on 5/09/2021

undesirable contaminants such as dioxins and dioxin-like PCBs, polycyclic aromatic hydrocarbons (PAHs) and pyrrolizidine alkaloids (PAs). were investigated

This thesis consists of six chapters.

Chapter 1 presents the general introduction, where a short background and description of the aims of the thesis are given. The introduction section also included description of key terms and the occurrence and characteristics of contaminants of concern, i.e. (synthetic) PDE-5 inhibitors, dioxins and dl-PCBs, PAHs, and PAs. The bioanalytical methods employed in this thesis were also introduced, i.e. the PDE-5 enzyme inhibition assay to detect the presence of PDE-5 inhibitors and the DR CALUX® bioassay to detect the presence of dioxins, dl-PCBs, and PAHs. Finally, the methods for risk assessment of consumers upon exposure to these contaminants were introduced in this chapter.

Chapter 2 through to **Chapter 5** described studies on the 4 main objectives of the thesis. These included the determination or prediction of the possible biological effect of the selected supplements by assessing their PDE-5 inhibition potentials using the PDE-Glo enzyme assay (Chapter 2). This was done to determine whether the supplements contained compounds with PDE-5 inhibition potentials and to select supplements of concern for further investigations. The aim of Chapter 3 was to investigate if the observed inhibition potentials of the supplements in the previous study were the result of adulterants (PDE-5i) added on purpose by producers to achieve the expected effects upon intake by consumers or whether they could be ascribed to natural plant constituents. This was achieved by using LC-MS/MS analysis. Eventually, a tiered approach, where the enzyme assay was applied in combination with LC-MS/MS and NMR analysis, resulted in the identification of a synthetic analogue that was initially not detected by the available analytical method, thus providing a proof-of-principle to detect novel PDE-5i when combining the bioassay with chemical analysis. The objective of Chapter 4 was to further screen the herbal supplements for the presence of less stable and more stable AhR-agonists (PAHs, PCDD/FS and dl-PCBs) using the DR CALUX bioassay in

combination with GC-HRMS analysis for confirmation of the observed responses. In Chapter 5, the presence of genotoxic and carcinogenic PAs was quantified. Chapter 6 is a general discussion where the results of the thesis were discussed whiles describing some challenges encountered during analysis and future perspectives.

Although about 90% of the supplements were able to inhibit PDE-5 enzyme activity at varying degrees (Chapter 2), the results of the chemical analysis pointed at the presence of adulterants (especially sildenafil) in about 60% of the supplements, intentionally added to products by producers with 13 (32.5 %) out of the 40 supplements showing concentrations at pharmacologically relevant levels (Chapter 3) and 6 (15 %) out of the 13 supplements even at toxicologically relevant levels. Furthermore, a screening test performed using the DR CALUX® bioassay revealed the presence of less stable AhR-agonists like PAHs in supplements rather than more stable AhR-agonists like dioxins and dl-PCBs. This was confirmed by the GC-HRMS analysis showing PAHs in some of the samples whilst very few samples contained dioxins and dl-PCBs. However, the calculated margin of exposure (MOE) for the Σ4PAHs based on the determined levels in the supplements and the estimated daily intakes were above 10 000, thus indicating low concern for consumer health and low priority for risk management actions. Similarly, the levels of PCDD/Fs and dl-PCBs would result in exposure far below the established tolerable weekly intake of 2 pg TEQ/kg bw/week thus also indicating low concern for consumer health. On the contrary, the concentration of PAs in 8 out of the 19 supplements containing PAs, would give rise to estimated daily intakes resulting in MOE levels below 10 000, thus indicative of a health concern upon daily life-long use of these products.

The presence of contaminants and especially added adulterants in the selected supplements compromises their purity and safety, the latter depending on the concentrations present. These findings are indicative of what is pertaining to the numerous herbal products available on the Ghanaian market. The practical approaches employed in this thesis, using a combination of a bioassay and chemical analysis to determine the presence and concentrations of added pharmaceuticals and

undesirable contaminants should be applicable in most laboratories. These approaches can therefore be applied in investigating other herbal products available on the Ghanaian market.

The adulteration of herbal products with active pharmaceutical ingredients and the presence of contaminants of concern continues to be a problem for regulatory agencies globally including Ghana. In the present thesis, the presence of intentionally added adulterants in the selected herbal supplements shed a light on on-going practices in Ghana and provides some answers to the numerous reported casualties and deaths of consumers of sexual enhancing herbal products sold on the Ghanaian markets.

Annex

Acknowledgements

About the author

List of publications

Overview of completed training activities

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ABOUT THE AUTHOR

Felicia Akuamoa was born on 15 April 1983 in Accra, Ghana. In 2002, she graduated from Aggrey Memorial Senior High in Cape Coast, where she pursued Home Economics (Food & Nutrition). She then proceeded to the University of Cape Coast in 2003, where she undertook her four years Bachelor of Education program in Home Economics (Food and Nutrition), and then to the School of Nuclear and Allied Sciences (University of Ghana) in 2010 for her Master in Philosophy (Food Irradiation Processing). In July 2017, she started her PhD journey at Wageningen University and Research in the Division of Toxicology and the Wageningen Food Safety Research, where she studied Food Toxicology. She has since participated in several activities and followed the Postgraduate Education in Toxicology, which will enable her to register as a European Toxicologist.

LIST OF PUBLICATIONS

Publications for this thesis

Akuamoa, F., Hoogenboom, R. L., Hamers, A., Rietjens, I. M., & Bovee, T. F. (2021). PDE-5 inhibitors in selected herbal supplements from the Ghanaian market for better erectile function as tested by a bioassay. *Toxicology in Vitro*, *73*, 105130. https://doi.org/10.1016/j.tiv.2021.105130

Felicia Akuamoa, Toine F.H. Bovee, Lilian Maro, Ruud van Dam, Sebastiaan Wesseling, Jacques Vervoort, Ivonne M.C.M. Rietjens, Ron L.A.P. Hoogenboom. Identification of phosphodiesterase type-5 (PDE-5) inhibitors in herbal supplements using a tiered-approach, and associated consumer risk. Accepted by *Food Additives and Contaminants* Manuscript ID TFAC-2021-411.

Felicia Akuamoa, Ron L.A.P. Hoogenboom, Yoran Weide, Guido van der Weg, Ivonne M.C.M. Rietjens, Toine F.H. Bovee. Presence and risks of polycyclic aromatic hydrocarbons, dioxins and dioxin-like PCBs in plant supplements as elucidated by a combined DR CALUX® bioassay and GC-HRMS based approach. (submitted).

Felicia Akuamoa, Toine F.H. Bovee, Ivonne M.C.M. Rietjens, Patrick P.J. Mulder, Ron L.A.P. Hoogenboom. Pyrrolizidine alkaloids in plant supplements marketed for improving sexual performance and associated health risk of consumers. (in preparation)

Other publications

- ***Akuamoa, F.***, Odamtten, G. T., & Kortei, N. K. (2020). Synergistic Effect of Gamma Irradiation and Proper Packaging for the Control of Insects in Smoked Shrimps (Penaeus notialis) from three different Water bodies in Ghana. *Cogent Food & Agriculture*, *6*(1), 1783176. https://doi.org/10.1080/23311932.2020.1783176
- ***Akuamoa, F.***, Odamtten, G. T., & Kortei, N. K. (2018). Nutritional and Shelf-life Studies of Dry Smoked and Gamma Irradiated Shrimps (Penaeus notialis) from three different Water Sources in Ghana. *Cogent Food & Agriculture*, *4*(1), 1505803. https://doi.org/10.1080/23311932.2018.1505803
- Kortei, N. K., Agyekum, A. A., ***Akuamoa, F.***, Baffour, V. K., & Alidu, H. W. (2019). Risk Assessment and Exposure to Levels of Naturally occurring Aflatoxins in some packaged Cereals and Cereal-based Foods consumed in Accra, Ghana. *Toxicology reports*, *6*, 34-41. https://doi.org/10.1016/j.toxrep.2018.11.012

- **Akuamoa, F.,** Odamtten, G. T., Kortei, N. K., & Agyekum, A. A. (2017). Comparative Studies on the Mineral Elemental Composition of Gamma Irradiated Smoked Shrimps (Penaeus notialis) from 3 Different Water Sources in Ghana. *Pharmaceutical and Biosciences Journal*, 43-50. https://doi.org/10.20510/ukjpb/5/i5/166556
- Crentsil Kofi Bempah, Akwasi Akomeah Agyekum, ***Felicia Akuamoa***, Samuel Frimpong, Achibold Buah-Kwofie. "Dietary Exposure to Chlorinated Pesticide Residues in Fruit and Vegetables from Ghanaian Markets". Journal of Food Composition and Analysis 46 (2016) 103–113. https://doi.org/10.1016/j.jfca.2015.12.001
- ***Akuamoa, F***., Odamtten, G. T., & Kortei, N. K. (2018). Impact of Gamma Radiation on the Microbiological Quality of Smoke Dry Shrimp (Penaeus notialis) from three different Water Sources in Ghana. *Cogent Food & Agriculture*, *4*(1), 1484200. https://doi.org/10.1080/23311932.2018.1484200

OVERVIEW OF COMPLETED TRAINING ACTIVITIES

Discipline specific courses

Course	Organized by	City	Year
Laboratory Animal Science	Postdoctoral Education in Toxicology	Utrecht	2018
Immunotoxicology	Postdoctoral Education in Toxicology	Utrecht	2018
Molecular Toxicology	Postdoctoral Education in Toxicology	Amsterdam	2018
Epidemiology	Postdoctoral Education in Toxicology	Utrecht	2018
Pathobiology	Postdoctoral Education in Toxicology	Utrecht	2019
Medical and Forensic Toxicology	Postdoctoral Education in Toxicology	Utrecht	2019
Organ Toxicology	Postdoctoral Education in Toxicology	Nijmegen	2020
Cell Toxicology	Postdoctoral Education in Toxicology	Leiden	2020
Food Toxicology	Wageningen University and Research	Wageningen	2017
Food Risk Assessment	Wageningen University and Research	Wageningen	2018
Environmental Toxicology	Wageningen University and Research	Wageningen	2018

General courses

Course	Organized by	City	Year
Scientific writing	Wageningen Graduate School	Wageningen	2019
Writing Grant Proposals	Wageningen Graduate School	Wageningen	2020
Posters and Pitching	Wageningen Graduate School	Wageningen	2020
Supervising BSc & MSc students	Wageningen Graduate School	Wageningen	2020
Philosophy and Ethics of Food Science and Technology	Graduate School of Biobased, Biomolecular, Food & Nutrition Sciences (VLAG)	Wageningen	2020
Reviewing Scientific Manuscript	Wageningen Graduate School	Wageningen	2021

Other activities

Course	Organized by	City	Year
Thesis Proposal	Division of Toxicology	Wageningen	2017
Attending scientific presentations	Division of Toxicology	Wageningen	2017-2021

Approved by the graduate school VLAG

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